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AP-2 in the Developing Chick Retina: the Search for Novel Target Genes and Function in Differentiation

by

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Christina Ann Sereda

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of Master of Science

in

Medical Sciences - Oncology

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Fall 2003

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September 23, 2003

ABSTRACT

Members of the AP-2 transcription factor family have been implicated in processes such as cell cycle control, apoptosis, and embryonic development. Previous research by our lab in the context of studying the regulation of the *Retinal-Fatty Acid Binding Protein* gene, has demonstrated that AP-2 is expressed in the amacrine and horizontal cells of the differentiating retina. We hypothesize that AP-2 is an important regulator of these cell types, and we contend that additional AP-2 target genes exist in the developing retina that have yet to be discovered. Using the chick as our model system, we are employing a number of approaches to identify these novel AP-2-regulated genes. As well, we are interested in the effects the misexpression of exogenous AP-2 has on the developmental potential of retinal cells. This work will provide insight into the complex pathways involved in retinal maturation.

DEDICATION

This thesis is dedicated to my younger brother, vice-skip, critic, and friend Dean Sereda for helping to make my university years both memorable and endurable.

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INDEX OF ABBREVIATIONS AND SYMBOLS

A	Adenosine
A	Amp(s)
AD	Activation domain
Amp	Ampicillin
AMV	Avian myeloblastosis virus
AP-2	Activator protein 2
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
bp	Base pair(s)
BPB	Bromophenol blue
BSA	Bovine serum albumin
C	Cytosine
C	Celcius
C-	Carboxyl
$CaCl_2$	Calcium chloride
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary DNA
CEF	Chick embryo fibroblasts
C_f	Final concentration
cfu	Colony forming unit
ChAT	Choline acetyltransferase
ChIP	Chromatin immunoprecipitation
Ci	Curie
CIAP	Calf intestinal alkaline phosphatase
cm	Centimetres
CO_2	Carbon dioxide
CPTS	Copper phthalocyanine tetrasulfonic acid
CsCl	Cesium chloride
d5	day five
dATP	2'-Deoxyadenosine-5'-triphosphate
DBD	DNA binding domain
DBH	Dopamine beta-hydroxylase
dCTP	2'-Deoxycytidine-5'-triphosphate
dGTP	2'-Deoxyguanosine-5'-triphosphate
dI	Deoxyinosine
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
d.p.c.	Days post coitum
DRG	Dorsal root ganglion

DTT	Dithiothreitol
dTTP	Thymidine-5'-triphosphate
ECL	Enhanced chemiluminesence
ED	Embryonic day
EDTA	Ethylenediamine tetraacetic acid
EGS	Ethylene glycol bis(succinimidylsuccinate)
EGTA	Ethylene glycol-bis-(2-aminoethylether)-N,N,N'-N'-tetraacetic acid
EMSA	Electrophoretic mobility shift assay
EtBr	Ethidium bromide
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
G1/S	Cell-cycle checkpoint between gap phase I and the start of DNA synthesis
g	Gram(s)
g	Gravity
G	Guanosine
GFP	Green fluorescent protein
GI	GenInfo identifier
GSH	Reduced glutathione
GST	Glutathione-s-transferase
HCHO	Formaldehyde
HCl	Hydrochloric acid
H&E	Hematoxylin and Eosin
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HH	Hamburger and Hamilton
H_2O	Water
HSH	Helix-span-helix
IHC	Immunohistochemistry
IP	Immunoprecipitation
IPTG	Isopropylthio-β-D-galactoside
ISH	*In situ* hybridization
kb	Kilobase pair(s)
KCl	Potassium chloride
kDa	Kilo Dalton
l	Litre
LB	Luria-Bertani medium
LiCl	Lithium chloride
LTR	Long terminal repeats
m	Milli-
m	Metre

M, mol	Molar
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
μ	Micro
N-	Amino
NaCl	Sodium chloride
β-NAD	Beta-nicotinamide-adenine dinucleotide
$NaHCO_3$	Sodium hydrogen carbonate
NaH_2PO_4	Sodium dihydrogen phosphate
NaoAc	Sodium acetate
NaOH	Sodium hydroxide
NE	Nuclear extract
NH_4oAc	Ammonium acetate
NLS	Nuclear localization signal
NP-40	Nonidet P-40
NT	Nick translation
O. C. T.	Optimal cutting temperature (medium)
OMIM	Online Mendelian inheritance in man
^{32}p	Radioactive phosphate
PARP	Poly(ADP)ribose polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Potential of hydrogen
PIPES	Piperazine-N, N'-bis(2-ethanesulfonic acid)
PK	Proteinase K
PMSF	Phenylmethanesulfonyl fluoride
pol	Polymerase
PPDA	P-phenylenediamine
PVP	Polyvinylpyrrolidone
RA	Retinoic acid
RCAS	Replication-Competent ASLV long terminal repeat (LTR) with a Splice acceptor
RCAN	Replication-Competent ASLV long terminal repeat (LTR) with no Splice acceptor
R-FABP	Retinal Fatty-Acid-Binding protein
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
SA	Splice acceptor
SD	Splice donor

SDS	Sodium dodecyl sulfate
sec	Second(s)
SSC	Standard saline citrate
SSH	Suppression subtractive hybridization
SUMO	Small ubiquitin-like modifier
SV40	Simian virus 40
T	Thymidine
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TE	Tris/EDTA
TEG	Tris-EDTA-Glucose
TEMED	N,N,N',N'-Tetramethylethylenediamine
TH	Tyrosine hydroxylase
Tm	Melting temperature
tRNA	Transfer RNA
TUNEL	TDT-mediated dUTP nick-end labelling assay
U	Unit(s)
UV	Ultraviolet [light]
V	Volt(s)
V_f	Final volume
WCE	Whole cell extracts
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactoside

CHAPTER ONE

Introduction

1.1.1. *Transcription Factors*

Carefully regulated programs of gene expression are necessary for the proliferation and differentiation of cells in all organisms during their development and for the proper functioning of all physiological processes. Transcription, or the copying of a sequence of DNA into RNA for subsequent protein synthesis or translation, is one multi-step process that mediates temporal and spatial control of gene expression. The events involved in transcription begin with the binding of a sequence-specific transcription factor to its recognition site in the promoter of a gene, upstream of the transcription start site (see Semenza, 1999). Then, other DNA-binding proteins and co-activators associate within the gene's promoter to disrupt nucleosomes and to allow the formation of the transcription initiation complex at the TATA box. Finally, the process culminates with promoter clearance and elongation of the transcript.

Transcription factors generally consist of two parts: a DNA binding domain (DBD) and an activation domain (AD). Based upon similarities observed in their DNA binding domains, many of these proteins have been grouped into families, such as the *HOX* family of genes. The majority of transcription factors fall into one of the following general structural categories: helix-loop-helix (*e.g.* MyoD), leucine zipper (*e.g.* Fos and Jun), zinc finger (*e.g.* WT1), and helix-turn-helix or homeodomain (*e.g.* Pax). It is through these motifs that transcription factors are able to recognize and create contacts with their associated DNA sequences (see Alberts *et al.*, 1997). Information encoded by the DNA base pairs, in the form of specific combinations of hydrogen bond donors, hydrogen bond acceptors, and methyl groups, is exposed via the major groove of the DNA double helix. Amino acids that are accessible as a result of the protein's tertiary structure are able to bind to the DNA with great affinity at these sites in the helix. The motifs found in the N-terminus, on the other hand, provide links between DNA and other protein co-factors. These co-factors, in turn, mediate contacts with the transcriptional machinery as they themselves are capable of protein:protein interactions (Semenza, 1999).

Transcription factors may be regulated directly at the level of protein synthesis. However, a host of other mechanisms are responsible for ensuring that transcription factors are activated at the appropriate times in a cell (reviewed in Lewin, 1997) and a few examples will be mentioned briefly here. For instance, phosphorylation and dephosphorylation are two post-translational modifications that have roles in regulating certain DNA binding proteins, such as the well-characterized p53 transcription factor. It has been shown that phosphorylation of serine 20 contributes to the activation of p53 by interfering with the binding site for Murine double minute clone 2 (Mdm2), a factor that negatively regulates p53 (Unger *et al.*, 1999). Dephosphorylation of serine 376 of p53, on the other hand, creates a recognition site for 14-3-3σ, a protein that increases p53's ability to bind DNA (Waterman *et al.*, 1998). This latter example shows that the association of an accessory protein or co-factor may be required in some cases for optimal transcription factor activity. Redox regulation is another way by which the activity of DNA-binding proteins can be controlled: proteins containing cysteine residues such as the Fos and Jun subunits of the AP-1 transcription factor, exhibit an increased capability to bind to DNA when in a reduced state (Sen and Packer, 1996). As well, a protein may be sequestered in an area of the cell where it is unable to mediate its effects. In particular, the nuclear localization signal (NLS) of the NF-κB transcription factor is masked when this protein is bound to its inhibitor, IκB (Liou and Baltimore, 1993). However, when IκB is inactivated through phosphorylation, NF-κB dissociates and enters the nucleus where it is able to mediate its effects.

Various pathologies that are associated with aberrant function of transcription factors demonstrate that it is critical for these proteins to maintain their proper roles in a cell. For example, levels of the p53 transcription factor increase in response to accumulated DNA damage from exposure to ionizing radiation or UV irradiation (reviewed by Levine, 1997). The target genes of p53 include *p21*, which encodes a cyclin-dependent kinase inhibitor that prevents the cell from progressing through the G1/S cell cycle checkpoint allowing time for the DNA to be repaired. The p53 protein also activates the *Bax* gene, whose protein induces apoptosis. Thus, in the absence of functional p53, DNA mutations can be propagated to daughter cells during mitosis. In fact, p53 mutations are associated with over 50% of human cancers. As well, Li-Fraumeni syndrome (OMIM: #151623) results from heterozygous germline mutations in the *p53* gene. This disorder is characterized by a predisposition to cancers, including osteosarcoma, leukemia, and breast carcinoma. Another example of a disorder that results from the loss of function of a transcription factor is Denys-Drash syndrome, which is characterized by Wilms' tumour, nephropathy, and genital abnormalities such as ambiguous genitalia (Mueller, 1994). Germ-line missense mutations in zinc fingers two and three of the WT1 protein were shown to interfere with its ability to bind DNA resulting in the observed phenotypic effects (Borel *et al.*, 1996).

Our studies have focused on the Activator Protein 2 or AP-2 transcription factor. Specifically, we are interested in the genes that AP-2 regulates in the context of retinal development. Using the chick as our model organism and a variety of techniques, we have endeavored to isolate novel target genes of AP-2 in the differentiating retina. As well, we are studying the effects misregulation of this transcription factor has on retinal cells during embryonic development. To achieve this goal, we are using a system of *in ovo* retroviral-mediated gene transfer into chick embryos.

1.1.2. *AP-2 Transcription Factors*

AP-2 refers to a family of dimeric transcription factors that are implicated in cell cycle control, apoptosis, and vertebrate development (reviewed in Hilger-Evershiem *et al.*, 2000). Mitchell *et al.* discovered the first of these proteins in 1987 by performing a DNA binding affinity assay using nuclear extract from HeLa cells and sequence from the SV40 enhancer. A 52-kDa protein was recovered, which bound *in vitro* to sequence from the SV40 and human metallothionein IIA promoters in DNase I protection assays. The consensus DNA binding site for AP-2 was later deduced to consist of the palindrome, $^{5'}$GCCNNNGGC$^{3'}$ (Williams and Tjian, 1991a). As well, it was determined that AP-2, which is comprised of 436 amino acids, arises from the splice product of seven exons that are located over 18 kb of genomic DNA (Bauer *et al.*, 1994).

Since then, additional AP-2 family members have been identified, bringing the total number of AP-2 proteins to four, designated AP-2α, -β (Moser *et al.*, 1995), -γ (also known as AP-2.2; Oulad-Abdelghani *et al.*, 1996), and -δ (Zhao *et al.*, 2001). These proteins exhibit most of their variation in their N-termini, which confers transactivation activity. Separate genes encode each one of these proteins, which map to different chromosomal loci. For instance, the gene for AP-2α maps to chromosome 6p22.3-24 by analysis of somatic cell hybrids and *in situ* hybridization (Gaynor *et al.*, 1991). Similarly, the *AP-2β* and *AP-2γ* genes have been shown to be located on chromosomes 6p12 and 20p13.2, respectively, through fluorescence *in situ* hybridization (Williamson *et al.*, 1996). While *AP-2δ* has been cloned and analyzed in mice, the human *AP-2δ* gene has not been characterized to date, although a predicted AP-2 gene distinct from *AP-2β* was reported to reside on 6p12-21.1 (Zhao *et al.*, 2001). Recently, Cheng *et al.* (2002) cloned a novel AP-2 gene from this same region that they named transcription factor AP-2β-like gene (*TFAP2BL1*). In light of this nomenclature, it is not clear if this gene is, in fact, *AP-2δ*, but a comparison of the reported sequences of mouse *AP-2δ* and *TFAP2BL1* show that they are virtually identical at the amino acid level. It has been speculated that the different AP-2 isoforms have arisen through duplication of an ancestral gene during evolution and that they allow for more flexibility in gene regulation (Moser *et al.*, 1995 and 1997).

In addition, a naturally occurring splice variant of AP-2α, AP-2B, has been characterized. A study by Buettner and colleagues (1993) showed that this protein differs from AP-2α in the last 70 amino acids of its C-terminus, which is responsible for DNA binding and dimerization (discussed below). It was demonstrated in CAT assays that activity from an AP-2 responsive promoter was diminished upon co-transfection of Schneider and PA-1 cells with AP-2α and AP-2B expression plasmids versus transfection of the AP-2α expression construct alone. Gel shift analyses also showed that AP-2B prevented AP-2α from binding *in vitro* to a DNA fragment containing an AP-2-consensus binding site and that AP-2B was incapable of binding to this site itself. Thus, AP-2B acts to negatively regulate the transcriptional activity of AP-2 in a dominant negative fashion.

1.1.3. *Structure of AP-2 Transcription Factors*

All AP-2 proteins consist of an N-terminal region that harbors the transactivation activity of AP-2 and a C-terminal domain, which is required for DNA binding and dimerization [Figure 1.1(a)]. The N-terminal region is rich in proline and glutamine residues, which have been shown to mediate transactivation activity and are present in a number of transcription factors (Gerber *et al.*, 1994). It is not yet known what the exact significance of these particular amino acids is with respect to conferring activity to a DNA binding protein

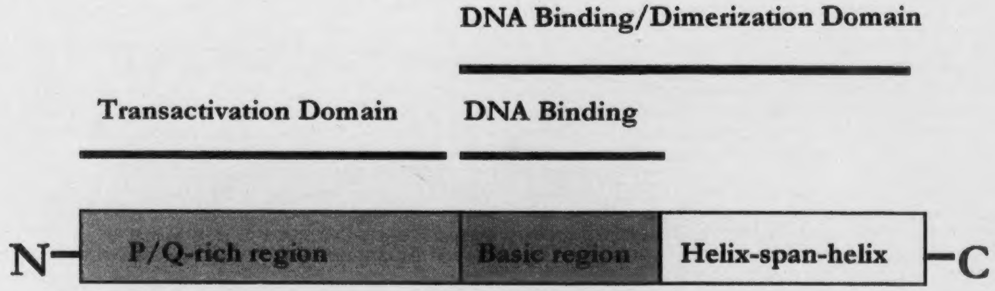
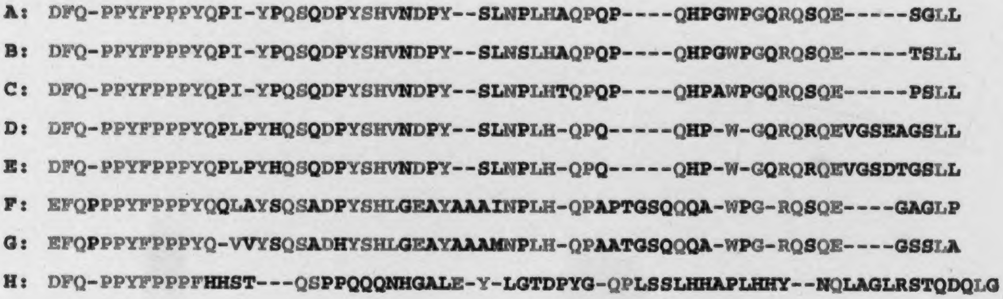


Figure 1.1(a): Schematic diagram of the general organization of all AP-2 transcription factors. N: amino terminus; C: carboxyl terminus. NB: Diagram is not to scale. Adapted from Hilger-Evershiem *et al.*, 2000.



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A:  DFQ-PPYFPPPYQPI-YPQSQDPYSHVNDPY--SLNPLHAQPQP----QHPGWPGQRQSQE-----SGLL
B:  DFQ-PPYFPPPYQPI-YPQSQDPYSHVNDPY--SLNSLHAQPQP----QHPGWPGQRQSQE-----TSLL
C:  DFQ-PPYFPPPYQPI-YPQSQDPYSHVNDPY--SLNPLHTQPQP----QHPAWPGQRQSQE-----PSLL
D:  DFQ-PPYFPPPYQPLPYHQSQDPYSHVNDPY--SLNPLH-QPQ-----QHP-W-GQRQRQEVGSEAGSLL
E:  DFQ-PPYFPPPYQPLPYHQSQDPYSHVNDPY--SLNPLH-QPQ-----QHP-W-GQRQRQEVGSDTGSLL
F:  EFQPPPYFPPPYQQLAYSQSADPYSHLGEAYAAAINPLH-QPAPTGSQQQA-WPG-RQSQE----GAGLP
G:  EFQPPPYFPPPYQ-VVYSQSADHYSHLGEAYAAAMNPLH-QPAATGSQQQA-WPG-RQSQE----GSSLA
H:  DFQ-PPYFPPPFHHST---QSPPQQQNHGALE-Y-LGTDPYG-QPLSSLHHAPLHHY--NQLAGLRSTQDQLG
```

Figure 1.1(b): Alignments of the sequences of various AP-2 proteins between position 52 and 108 showing the similarity between these isoforms in their ADs. The conserved residues are shown in pink. The dashes have been inserted to preserve the alignment, and represent residues that are missing in one protein when compared to another. A: human AP-2α; B: *Xenopus* AP-2α ; C: chicken AP-2α ; D: murine AP-2β; E: chicken AP-2β; F: human AP-2γ; G: murine AP-2γ; H: *Drosophila* AP-2α. Figure adapted from Wankhade *et al.*, (2000).

(Wankhade *et al.*, 2000). Williams and Tjian (1991a) showed that a segment of AP-2's N-terminus containing these amino acids was capable of activating transcription of a reporter construct in a CAT assay by attaching this region to the GAL4 DNA binding domain. They postulated that proline and glutamine residues could provide a framework that exposes other critical residues that interact with general transcription factors or that these amino acids provide a link to the RNA Polymerase II transcription machinery. More recently, Wankhade *et al.* (2000) performed a detailed mutational analysis of the residues located in the N-terminus of AP-2. They discovered that the region between the aspartic acid at position 52 and the leucines at positions 107 and/or 108 were critical for enabling transactivation in CAT reporter assays. As well, this group demonstrated that other residues, in addition to the prolines and glutamines just mentioned, seem to be essential for proper functioning of AP-2. Mutation of a tyrosine and a phenylalanine residue to prolines actually reduced transcriptional activity of the N-terminal fragment. Notably, most intra- and interspecies variation (discussed below) between AP-2 molecules occurs in the N-terminal domain, yet these isotypes display strict conservation in the region located between position 52 and 108 [Figure 1.1(b)].

Conversely, AP-2 proteins show much higher conservation along the *entire* length of their C-terminal domains. As previously mentioned, the C-terminus of AP-2 mediates DNA binding and dimerization. EMSA analyses were performed with AP-2 mutants containing various N-terminal and C-terminal deletions, and in this manner, it was established that this region was necessary for DNA binding (Williams and Tjian, 1991b). This domain of approximately 200 amino acids can be sub-divided into two regions, a segment containing a helix-span-helix (HSH) motif, consisting of two alpha helices separated by 82 amino acids, and a region of net basic charge. Further experiments have defined the specific functions of these two domains. Since AP-2 recognizes a palindromic sequence, it was speculated that it forms a dimer. Co-immunoprecipitations were performed to determine which of the deletion mutants were capable of binding to wild-type AP-2. Mutants with C-terminal deletions, in particular those with deletions in the HSH motif, were incapable of forming dimers with full-length AP-2, while mutants only missing portions of the N-terminus were recovered in the immunoprecipitation reaction (Williams and Tjian, 1991a&b). As well, it was observed that mutants that did not possess dimerization capabilities also did not bind DNA in EMSA reactions. The basic region was established as essential to DNA binding, based on the observation that a protein fragment containing the HSH alone could not bind DNA, even though it retained the ability to dimerize. Thus, dimerization, mediated by the HSH motif, is a prerequisite for DNA binding, while DNA:protein interactions are dependent on the basic region.

AP-2 proteins have been demonstrated to be capable of binding to DNA as either homo- or heterodimers. For instance, Bosher *et al.* (1996) performed EMSA analyses with sequence from the AP-2-regulated *c-erbB-2* promoter and different combinations of *in vitro* translated AP-2α, -β, and -γ. In order to distinguish the different bands formed on a gel, some of the AP-2 molecules had N-terminal deletions. These modifications do not affect the DNA binding and dimerization properties of the proteins as already discussed, but change the mobility of the shifted complexes. In fact, shifted bands corresponding to all possible combinations of heterodimeric complexes were observed on the gels. The roles of these AP-2 heterodimers *in vivo* have not been clearly defined to date.

1.1.4. *AP-2 Molecules Across Species*

As previously mentioned, genes encoding AP-2 proteins exist in other species besides humans, namely, mouse (Moser *et al.*, 1995), chicken (Shen *et al.*, 1997), sheep

(Limesand and Anthony, 2001), *Xenopus* (Winning *et al.*, 1991), *Drosophila* (Monge and Mitchell, 1998), and amphioxus and lamprey (Meulemans and Bronner-Fraser, 2002). As well, genes with AP-2 binding sites in their promoters are reported in the cow (Kuss *et al.*, 2003) and pig (Baskin *et al.*, 1997). In many cases, these AP-2 proteins show remarkable interspecies conservation. For instance, Wankhade and colleagues (2000) performed sequence alignments on various AP-2 proteins, and found that murine AP-2β is 100% identical at the amino acid level to human AP-2β in the two domains. As well, *Xenopus* AP-2α protein is 87.8% identical to human AP-2α in the activation domain (AD) and 98% identical in the DNA binding domain (DBD). On the other hand, *Drosophila* AP-2α (originally named DAP-2) exhibits quite a lot less conservation with its human counterpart with only 28.1% and 67.8% identity in the AD and DBD, respectively. Despite the low degree of conservation, however, Bauer *et al.* (1998) showed that DAP-2 was functionally conserved, as it activated transcription from an AP-2 responsive promoter in human cells. DAP-2 also retained the amino acid sequence DFQPPYFPPP, which is found in the N-terminus of all AP-2 proteins [Figure 1.1(b)].

1.1.5. *Analyses of AP-2 Expression*

Various studies have focused on defining the specific tissues and organs in which *AP-2* is expressed during development. A study by Mitchell *et al.* (1991) for example, revealed through *in situ* hybridizations of mouse embryo sections that *AP-2α* expression begins around 8.5 d.p.c. in the neural folds and head mesenchyme. Notably, this is the location of the earliest neural crest cells in the embryo. Analyses of later stages revealed the presence of *AP-2α* transcripts in a variety of structures derived from neural crest cells such as the dorsal root ganglion (DRG) and facial and branchial arch mesenchyme. *AP-2α* expression was also observed in the epidermal ectoderm and mesometanephric regions.

A second study by Moser *et al.* (1997) also used *in situ* hybridization with gene specific probes to compare the expression patterns of *AP-2α* and *AP-2β* at various developmental stages in the mouse. This group found that *AP-2α* and *AP-2β* expression are virtually indistinguishable from 8 to 10 d.p.c. However, starting around 11 d.p.c., differences in expression were observed between the two genes. For instance, *AP-2α* expression was virtually absent in the midbrain, while *AP-2β* mRNA was present in significant amounts in this structure. As well, the cranial and dorsal root ganglia (DRG) were positive for *AP-2α* but not *AP-2β*. While the later stage kidney was positive for both forms of *AP-2*, *AP-2α* was expressed predominantly in the proximal tubule while *AP-2β* transcripts were localized to the distal tubule and collecting duct. *AP-2α* expression was unique to the tooth germs, limb buds, and Moll's and Meibom's glands, whereas only *AP-2β* signal was observed in the cornea, adrenal medulla, and mesencephalon.

A third study examined the expression of *AP-2γ* during mouse embryogenesis. Chazaud *et al.* showed that as early as 7.0 d.p.c., *AP-2γ* transcripts were evident, although these were localized in the extra-embryonic tissues. No *AP-2γ* expression was observed in the embryo itself until 7.5 d.p.c. Like *AP-2α*, *AP-2γ* was expressed in the head fold mesenchyme. However, *AP-2γ* was noticeably absent from the DRG. As well, *AP-2γ* signal was visualized in the forebrain, in contrast to *AP-2α*, which was not found in this region. *AP-2γ* transcripts were also present in the facial region (Chazaud *et al.*, 1996). Taken together, these results suggest that the various AP-2 genes probably have some identical functions, but may have different roles and target dissimilar genes due to their unique patterns of localization in various structures during embryonic development.

1.1.6. *AP-2 Knockouts*

To date, knockout mice have been generated for three of the four forms of AP-2, in order to further elucidate the functions of the individual members of the AP-2 transcription factor family. In some cases, the knockouts confirmed the results obtained in the *in situ* hybridization experiments. In 1996, Schorle *et al.* and Zhang *et al.* reported obtaining AP-2α -/- mice by targeted disruption of exons 5 and 6, respectively, which are responsible for dimerization and thus proper functioning of AP-2α. The mice generated in the two studies died perinatally as a result of their deleterious phenotypes, which included cranio-abdominoschisis. Cranio-abdominoschisis is a phenomenon in which the cranial portion of the neural tube and the body wall fails to close, resulting in the brain and other internal organs developing evertedly outside these embryos. Because the neural tube and body wall remain open, mid-line structures such as the mandibular and nasal prominences are displaced laterally. AP-2α-/- mice were also observed to lack external eyes and ears. In addition, skeletal preparations of embryos showed that the axial skeletons of the null mice were scoliotic when compared to their wild-type littermates and more strikingly, these mice displayed the absence of a formed cranium. Miscellaneous limb defects such as a missing radius in the forearm were observed, although these varied in severity. In a follow-up study, Nottoli *et al.* (1998) attempted to generate chimeric mice composed of AP-2α-/- and wild-type cells to examine the individual developmental pathways influenced by AP-2. These mice demonstrated that AP-2 is necessary for proper craniofacial formation, as many embryos displayed cleft lips and palates. As well, a requirement for AP-2 in eye formation was shown: possible roles for AP-2 in eye development were masked in the AP-2α knockout embryos due to their severe phenotype. In the chimeras, eyelids were commonly missing, and the eyes were often misshapen and embedded in the head.

AP-2β-/- mice, in contrast, did not exhibit the severe morphological defects manifested in the AP-2α-/- mice, providing additional evidence that these proteins have markedly different roles during development. However, like the AP-2α knockout mice, these mice were not viable and died perinatally (Moser *et al.*, 1997). In analyses of the AP-2β knockouts most of the organs appeared normal with the exception of the kidneys, which contained numerous microscopic cysts. These cysts appeared to form late in embryonic development, as examination of embryos at various stages showed that AP-2β null embryos had normal kidneys up to and including 15.5 d.p.c. TUNEL analyses revealed that apoptotic cell death was increased eight fold in the kidneys of knockout animals versus wild-type mice. It was postulated that AP-2 was responsible for suppressing c-myc-induced apoptosis and that its loss led to this increase in cell death. Another group had previously demonstrated that AP-2 negatively regulates Myc, both by directly binding to it and by competing for binding at the promoter regions in Myc-regulated genes, where AP-2 binding sequences are found adjacent to the Myc-response elements (Gaubatz, 1995). To support their hypothesis, Moser *et al.* (1997) examined DNA fragmentation, a measure of apoptosis, and found that it was significantly inhibited in PA-1 cells that were co-transfected with c-myc and AP-2β expression vectors versus cells transfected with the c-myc vector alone. Incidentally, PA-1 cells contain low levels of endogenous AP-2. As in the case of AP-2α, the AP-2β knockout mice were generated by targeted disruption of an exon responsible for DNA binding and dimeration of the protein, exon 4.

Lastly, knockout mice were recently generated for the third AP-2 gene, *AP-2γ*. These mice proved to be embryonic lethal as they failed to survive beyond 8.5 d.p.c. (Auman *et al.*, 2002, Werling and Schorle, 2002). The extra-embryonic tissues of these embryos were observed to be disorganized and underdeveloped. As well, these embryos were delayed in their development and the maternal-embryonic interfaces were disrupted. Auman *et al.* (2002) also generated AP-2γ chimeric mice to study further the consequences of AP-2γ disruption in the embryo itself, in isolation from its effects on the extra-embryonic tissues. Chimeric mice possessing extra-embryonic tissues that were derived from the wild-type host blastocyst, yet containing a substantial portion of AP-2γ null cells in the embryo proper, survived until they were sacrificed approximately one year later. Thus, it was concluded that while AP-2γ is essential for the development of extra-embryonic tissues, it is not critical for later development of the embryo.

1.1.7. *Char Syndrome*

AP-2, specifically AP-2β[*], has been implicated in a human genetic disorder, Char syndrome, which displays autosomal dominant inheritance (OMIM: 169100). One characteristic feature of Char syndrome is patent ductus arteriosus, a form of congenital heart disease in which the ductus arteriosus, the duct that directs blood away from the lungs during fetal development, fails to close at birth. Patients afflicted with Char syndrome also display distinct facial features such as a short philtrum, flattened nasal bridge, upturned nares, and prominent lips. Occasionally, these people lack the fifth middle phalanges in their hands (Satoda *et al.*, 1999; Zannolli *et al.*, 2000).

By examining the pedigrees of two large families and performing linkage analysis with a variety of markers, Satoda *et al.* (1999) mapped the critical region containing the gene responsible for Char syndrome to chromosome 6p12-p21. In a subsequent study, this group performed PCR analyses on DNA samples from one of the families using primers designed to amplify the coding exons of AP-2β. Sequencing of the products revealed a C→A transversion in exon five in the genes of affected individuals. This point mutation results in a substitution of an aspartic acid for an alanine residue at codon 264. Analyses of samples from a second family showed that a C→T transition had occurred in Char patients that changed an arginine to a cysteine residue at position 289. Both of these mutations are located in the DNA binding and dimerization domain of AP-2β. The products from these mutant genes were shown to be capable of forming dimers by cross-linking recombinant protein with ethylene glycol bis(succinimidylsuccinate) (EGS) and performing electrophoresis under denaturing conditions: cross-linked AP-2β mutant proteins formed a 100 kDa protein, like wild type AP-2α and AP-2β, on a gel. While these proteins are capable of forming dimers, EMSA gels revealed that mutant AP-2β is able to dimerize with AP-2α, and this heterodimer is unable to bind to DNA. Because the mutant AP-2β monomers are capable of forming heterodimers with the functional AP-2 monomers, they act in a dominant negative manner (Satoda *et al.*, 2000).

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[*] Human AP-2β is sometimes referred to as TFAP-2B, not to be confused with dominant negative AP-2B.

1.1.8. *Regulation of AP-2*

Studies have revealed that AP-2 is regulated in a number of different ways. For instance, Bauer *et al.* (1994) showed that the AP-2 protein stimulates transcription of its own gene (*i.e.* it is capable of autoregulation). This group, in trying to deduce the genomic structure of AP-2, found an AP-2 consensus site in its promoter. Footprint analyses, gel mobility shift assays, and transfection experiments with reporter and expression plasmids showed that the AP-2 protein binds to the AP-2 gene promoter and transactivates it (Bauer *et al.*, 1994). Kannan *et al.* (1994) demonstrated that N-ras-transformed PA-1 cells exhibited higher levels of AP-2 transcripts on Northern blots than non-transformed PA-1 cells. Surprisingly, the cells with higher amounts of AP-2 mRNA were much less capable of transactivating an AP-2 reporter construct in CAT assays. By fusing the N-terminal domain of AP-2 to the GAL4 DBD, this group showed that it was, in fact, the N-terminus that was responsible for this observed self-interference. Transfection of high amounts of this fusion protein squelched activation of the GAL4 reporter. It was proposed that excessive amounts of AP-2 depleted limited amounts of other factors necessary for its activity (discussed below). In addition, retinoic acid (RA), a form of vitamin A, appears to induce transient expression of AP-2. Lüsher *et al.* (1989) performed RNase protection assays on RNA isolated from NT-2 cells following the addition of RA to examine the resulting levels of AP-2 mRNA. They observed that the levels of AP-2 transcripts increased for two to three days following the introduction of RA. As well, Western immunoblotting detected AP-2 protein in cells grown in the presence of RA but not in cells cultured in its absence. This group also set up nuclear run-on assays with nuclei from NT-2 cells grown in the presence or absence of RA. They found a stronger AP-2 signal on a filter probed with transcripts from the RA treated cells versus the untreated cells, and they concluded that induction of AP-2 mRNA was occurring at the level of transcription.

Post-translational modifications have been associated with AP-2. In particular, Eloranta and Hurst (2002) performed a yeast two-hybrid assay with AP-2γ and they isolated the UBC9 protein, a SUMO-conjugating enzyme. SUMO is a small ubiquitin-like modifier that becomes conjugated to a target protein through an isopeptide linkage to a lysine residue within a specific amino acid motif. Whole cell extracts were prepared from MCF7 cells in a manner as to prevent isopeptidases from cleaving the SUMO moiety from the proteins. Western immunoblots of these extracts following SDS-PAGE revealed a higher molecular weight band corresponding to the sumolated form of AP-2. A subsequent reporter assay revealed that overexpression of SUMO via transfection of an expression vector resulted in decreased activity of both exogenous and endogenous AP-2 protein in various cell types. The precise manner in which SUMO affects AP-2's transactivation activity is not known, but since the modified lysine was shown to be present in the N-terminus, it was speculated that sumolation weakens the interactions between AP-2 and its cofactors. Park and Kim (1993) also showed that AP-2 is phosphorylated *in vivo* by cAMP-dependent protein kinase. It is not known what the significance of this modification is, but EMSA analyses revealed that the ability of phosphorylated AP-2 to bind to DNA was unaffected.

As well, a number of AP-2 co-activators have been identified to date through various methods. Campillos *et al.* (2003) performed AP-2α affinity chromatography with rat nuclear extract and isolated the DEK oncoprotein. A follow-up transfection experiment of low AP-2-expressing HepG2 cells with expression vectors for DEK and AP-2 revealed that DEK increases AP-2's transactivation activity in a dose-responsive manner. Other experiments by Kannan *et al.* (1999) identified polyADP-ribose polymerase (PARP) as another AP-2 co-activator. PARP was retrieved in a GST-AP-2 binding assay, and this molecule was shown to restore the transactivation activity of AP-2 in cells where it was exhibiting transcriptional self-interference. This group isolated another protein in the same manner, PC4, which also physically interacted with AP-2 and relieved its self-interference in reporter assays (Kannan and Tainsky, 1999). It was speculated that PARP and PC4 are present in finite amounts in the cell, and once their levels are depleted, self-interference of AP-2 is then observed. Finally, Bamforth *et al.* (2001) reported the generation of Cited2-/- mice. Interestingly, these mice displayed phenotypic characteristics reminiscent of AP-2-/- mice such as incomplete neural tube closure and neural crest defects. *In vitro* binding assays showed that AP-2 binds to GST-Cited2. Thus, this group hypothesized that Cited2 was a necessary requirement for

AP-2 functioning, and they demonstrated through transfection experiments that Cited2 increased AP-2's ability to stimulate an AP-2 responsive promoter.

1.1.9. *AP-2 Regulated Genes*

A vast number of genes are reported in the literature to be regulated, both positively and negatively, by AP-2 transcription factors. A partial list of the genes that are activated by AP-2 includes *p21* (Zeng *et al.*, 1997), estrogen receptor (McPherson *et al.*, 1997), *c-KIT* (Huang *et al.*, 1998), dopamine β-hydroxylase (Greco *et al.*, 1995), transforming growth factor-α (Wang *et al.*, 1997), HTLV-1 (Muchardt *et al.*, 1992), insulin-like growth factor binding-5 (Duan and Clemmons, 1995), *Rb2/p130* (a member of the Retinoblastoma protein family, Paggi *et al.*, 2001), and *bcl-2* and E-cadherin (Decary *et al.*, 2002), in addition to human metallothionein IIA (Mitchell *et al.*, 1987) and *c-erbB-2* (Bosher *et al.*, 1996), genes already mentioned above. Genes reported to be negatively regulated by AP-2 include *MCAM/MUC18* (Jean *et al.*, 1998) and *C/EBP-α* (Jiang *et al.*, 1998). As well, our lab has reported that AP-2 negatively regulates the retinal fatty acid binding protein (R-FABP) gene (Bisgrove and Godbout, 1999). Given that AP-2 has been associated with such a large number of diverse genes, it is evident that it has an essential role in an organism. The roles of AP-2 in cancer will be discussed in the following section.

1.1.10. *AP-2 and Cancer*

Numerous studies to date have revealed that AP-2 proteins appear to have a role in a variety of malignancies. For instance, Zeng *et al.* (1997) demonstrated that AP-2 expression has an impact on the proliferation of human colon adenocarcinoma cells. This group co-transfected SW480 adenocarcinoma cells with AP-2 and GFP expression plasmids. The latter construct was used to monitor the transfected cells and the subsequent formation of cell aggregates, which was evidence of cell division. The AP-2-transfected cells failed to form increased numbers of these aggregates when compared to cells transfected with a control vector. Also, AP-2 overexpression in the SW480 transfected cells significantly inhibited their ability to form stable colonies compared to the control-transfected cells. A possible explanation for these observations is that expression of the cyclin dependent kinase inhibitor, *p21*, is influenced by AP-2. It was shown in this study by immunohistochemical and Western blot analyses that p21 levels were elevated in the AP-2-transfected cells (Zeng *et al.*, 1997). A subsequent study by Ropponen *et al.* (2001) examined the expression of AP-2α, -β, and -γ in colorectal adenoma and adenocarcinoma samples by immunohistochemistry (IHC). Their results showed that AP-2α protein expression was reduced in high-grade colorectal carcinomas when compared to low-grade tumours. However, *in situ* hybridization (ISH) analyses showed that in a number of cases, AP-2α mRNA expression was not concomitantly down-regulated in these cells. This group proposed that either post-transcriptional regulatory mechanisms or the repression of translation initiation were responsible for controlling AP-2α protein levels in these cases.

A study by Anttila *et al.* (2000) reached a similar conclusion regarding AP-2α regulation in cancer. This group found a significant correlation between cytoplasmic AP-2α expression and survival in epithelial ovarian carcinomas. IHC analyses revealed that the presence of AP-2α protein in ovarian carcinoma tumour sections was a favorable prognostic indicator for this disease. ISH and RT-PCR experiments, though, showed that AP-2α transcripts were present in approximately half of the cases that lacked detectable AP-2α protein, leading to the suggestion that post-transcriptional regulatory controls were inhibiting this protein's expression.

AP-2α was also shown to be associated with prostate cancer. Ruiz *et al.* (2001) demonstrated through immunofluorescent staining of clinical samples that AP-2α protein was not expressed in prostate cancer specimens whereas it was present in normal prostate epithelium. They proposed that loss of AP-2α is a critical event in prostate cancer development, possibly resulting in the deregulation of other genes involved in the later stages of this disease.

There are other cancers in which the role of AP-2 has been more extensively characterized. For instance, a number of reports have implicated the loss of AP-2 in melanoma progression (reviewed by Bar-Eli, 1997 and 2001). Gershenwald *et al.* (2001) transfected SB-2 melanoma cells, which are typically non-metastatic, with a construct encoding the dominant negative AP-2B protein. These transfected cells with altered AP-2 activity formed significantly larger tumours than control SB-2 cells upon subcutaneous injection into nude mice, and they were metastatic. The deregulation of the matrix metalloproteinase-2 (*MMP-2*) gene, which contains an AP-2 binding site in its promoter, was proposed to be the cause of the observed increase in tumourigenicity. MMPs are significant enzymes in cancer, as they mediate basement membrane degradation, an event that precedes the occurrence of metastases (Chambers and Hill, 1998). *MMP-2* mRNA levels were higher in AP-2B-transfected cells in comparison to control cells with low tumourigenicity (Gershenwald *et al.*, 2001). A zymogen analysis demonstrated an increase in MMP-2 activity in AP-2B-transfected SB-2 cells, which were more able to penetrate through a Matrigel-coated filter than non-transfected cells. As well, co-transfection of an *MMP-2* CAT reporter construct with an AP-2 expression vector into the AP-2B transfected cells revealed a dose-dependent reduction in reporter activity in the presence of AP-2. The authors concluded from these results that AP-2 negatively regulates *MMP-2* expression. As a consequence, MMP-2 is upregulated following the loss of AP-2, which seems to be an important factor in melanoma progression.

There are other pathways involving AP-2, besides the one just described, which appear to be involved in melanoma progression. The c-KIT protein, for example, is a tyrosine kinase receptor, and its loss appears to be correlated with the absence of AP-2 in metastatic melanoma cells. The *c-KIT* promoter contains three predicted AP-2 binding sites, and a fragment from this region binds recombinant AP-2 protein (Huang *et al.*, 1998). A direct correlation was also observed between *AP-2* and *c-KIT* expression. Northern blots were prepared using mRNA from various melanoma cell lines and cells that expressed *AP-2* displayed a positive signal for *c-KIT* and vice versa. In addition, upon stable transfection with an AP-2 expression construct, melanoma cells that lacked both AP-2 and c-KIT protein exhibited re-expression of c-KIT protein. These transfected cells were significantly less capable of *in vivo* tumour formation in the nude mouse model than the non-transfected cells. Furthermore, a TUNEL assay showed that upon exposure to stem cell factor (SCF), a molecule produced by keratinocytes, c-KIT-expressing melanoma cells undergo apoptosis (Huang *et al.*, 1996). In light of these data, it has been proposed that the loss of c-KIT expression following the prior loss of AP-2 expression, allows melanoma cells to escape SCF-mediated apoptosis and proliferate.

AP-2 also appears to be responsible for the regulation of melanoma cell adhesion molecule (MCAM/MUC18) in the context of melanoma progression. MCAM/MUC18 is a cell-surface glycoprotein that mediates cell-cell or cell-matrix interactions and it is present in highly metastatic cells. Jean *et al.* (1998) conducted experiments that showed AP-2 binds to the *MCAM/MUC18* promoter *in vitro*, and in transfection studies, AP-2 down-regulates activity from an MCAM/MUC18 CAT reporter construct in a dose-dependent manner. As well, cells transfected with AP-2 showed a reduction in MCAM/MUC18 protein levels compared to non-transfected cells, and these transfected cells were less tumourigenic *in vivo* than non-transfected control cells. In contrast, a second group analyzed various melanoma cell lines and demonstrated that AP-2 is present in cell lines that both contain and lack MCAM/MUC18 expression (Mintz-Weber and Johnson, 2000). This finding would suggest that the loss of AP-2 alone is not required for the upregulation of MCAM/MUC18. Clearly, before final conclusions can be reached on the role of AP-2 in regulation of this molecule, more investigation needs to be undertaken.

Finally, several studies have arrived at the conclusion that AP-2 plays a substantial role in mammary carcinomas. In particular, two molecules associated with breast cancer, estrogen receptor (ER) and insulin-like growth factor I (IGF-IR), were shown to be AP-2-regulated. ER-positive breast cancers are generally associated with a favorable prognosis, as the response rates to hormonal therapy and the survival rates of these patients are higher when compared to cancers of ER-negative status (Goss and Tye, 1998). Studies of the ER promoter revealed a stretch of sequence with similarity to the AP-2 consensus site. The protein that binds to this site was identified as AP-2γ following its isolation and sequencing (McPherson *et al.*, 1997). In an EMSA analysis, a complex of DNA from the *ER* promoter and protein from MCF nuclear extracts formed, which supershifted upon the addition of anti-AP-2 antibody. This indicated that the protein under examination was an AP-2-like factor. As well, both AP-2α and AP-2γ activated the ER promoter in an *in vitro* reporter assay (McPherson and Weigel, 1999). Thus, it appears that AP-2 stimulates transcription from the ER promoter. In contrast to ER, overexpression of IGF-IR is associated with poor survival and this molecule has an anti-apoptotic effect. A study by Turner and associates showed through IHC analysis of breast cancer tissue samples that the absence of AP-2γ correlates with a reduction in IGF-IR expression. An AP-2 site in the *IGF-IR* promoter was also identified, which was shown to bind AP-2. These observations suggest that AP-2 represses *IGF-IR*; however, additional experiments such as transfection studies with a reporter gene linked to the IGF-IR promoter were not performed to confirm this hypothesis. In their immunohistochemical analyses, this group demonstrated that cells positive for both AP-2α and AP-2γ were more likely to express *c-erbB-2*, discussed next.

C-erbB-2 (also known as *HER2/neu*) encodes a 185-kDa tyrosine kinase receptor, and the presence of this protein is indicative of a poor prognosis for breast cancer patients. Several studies have demonstrated the involvement of AP-2 in the regulation of this gene. For instance, Bosher *et al.* (1995, 1996) showed that AP-2 binds to the *c-erbB-2* promoter and that a complex of *c-erbB-2* promoter DNA and recombinant AP-2 protein is supershifted by an anti-AP-2 antibody. In addition, a Western blot analysis revealed that while over-expressing c-erbB-2 cell lines contained AP-2 protein, no AP-2 was detected in low-expressing c-erbB-2 lines. In an *in vitro* CAT reporter assay, AP-2α, -β, and -γ were all capable of mediating activity from a fragment of the *c-erbB-2* promoter. More recently, however, Gee *et al.* (1999) have demonstrated, by immunostaining of breast cancer tissue samples with an antibody that recognizes both AP-2α and -β, that AP-2α/β expression is inversely correlated with c-erbB-2 expression. The role of AP-2γ was unfortunately not examined in this study, so it is difficult to speculate as to why these experiments produced contradictory results. Thus, more studies are necessary to establish the role of AP-2 in breast cancer, as it appears to be rather complex, especially in light of a recent report that examines the roles of estrogen and AP-2 in the regulation of c-erbB-2. This study by Perissi *et al.* (2000) showed that estrogen down-regulates c-erbB-2, and deletion/mutation studies revealed that an intact AP-2 binding site in the *c-erbB-2* promoter was essential for this repression by estrogen.

1.2. *Ocular Development*

1.2.1. *The Eye*

Eye development in vertebrates is a complicated process that requires the coordinated efforts of a multitude of factors and signals. The main events involved in eye formation will be discussed briefly here. The eye is actually an extension of the brain/central nervous system, and its formation begins with a folding or evagination of the diencephalon as the neural tube is closing to create the optic pits. The optic pits continue to protrude outwards to form the optic vesicles, which, in turn, make contact with the overlying surface ectoderm (see Figure 1.2.1). Inductive signals are exchanged between these two tissues, and a region of the surface ectoderm thickens to form the lens placode. These cells then invaginate to create the lens vesicle, which detaches from the remainder of the surface ectoderm to become the presumptive lens. As the presumptive lens is forming, the optic vesicle invaginates to form a double-layered optic cup. The outer portion of the optic cup is destined to become the retinal pigmented epithelium (RPE) while the inner region forms the neural retina (Sivak and Sivak, 2000; Chow and Lang, 2001).

1.2.2. *The Retina*

The retina is a tissue that lines the back of the eye. Its fundamental purpose is to convert light energy into nerve impulses through the process of phototransduction. The neural retina consists of various cell types, which are organized in a lamellar fashion. It has been established that a common progenitor cell gives rise to the neuronal cells of the retina, which include the amacrine, horizontal, bipolar, photoreceptor, ganglion, and interplexiform cells, and one type of glial cell, the Müller glial cell (Turner and Cepko, 1987). Individual classes of cells may be comprised of many different types: photoreceptors include rods and cones, while nearly two dozen subsets of amacrine cells have been identified in the rabbit

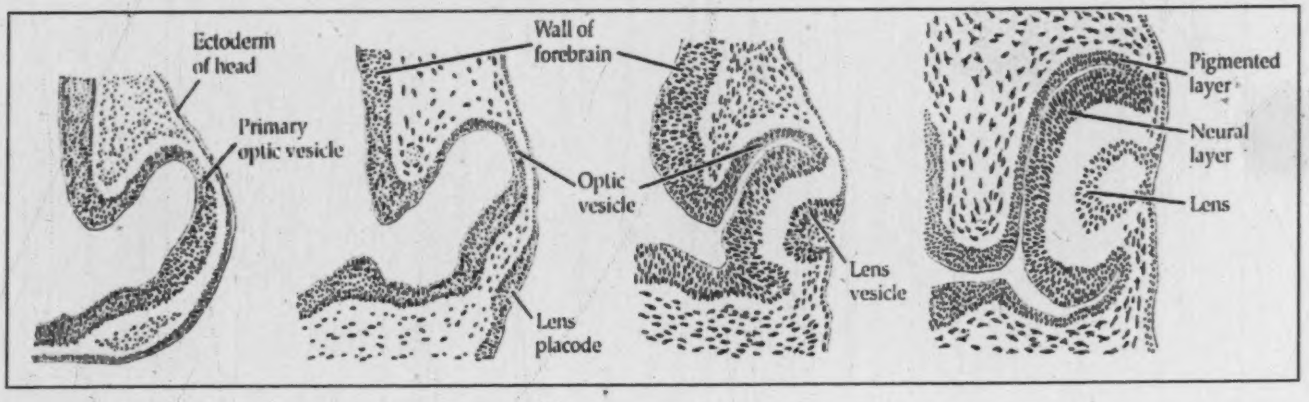

Figure 1.2.1: Development of the vertebrate eye. See text for details (illustration from Mann, 1964).

retina (reviewed by Livesay and Cepko, 2001). Figure 1.2.2 depicts the distribution of the various cells in the retina.

1.2.3. *The Chick as a Model Organism for Retinal Studies*

We are using the chick to study the role of the AP-2 transcription factor in the developing retina. The chick is amenable for retinal studies because eye development is well conserved among vertebrates, and fertilized eggs are easy to obtain, house, and manipulate. As well, the eyes of chick embryos are quite large in proportion to their bodies, especially at early embryonic stages. Thus, less effort is required to obtain adequate amounts of tissue for experiments in comparison to other organisms such as mice. The development of the chick has also been extensively characterized. In particular, Hamburger and Hamilton constructed a descriptive staging system in 1951 that is still widely used by embryologists today. With respect to early eye development, optic vesicles appear in the chick at Hamburger and Hamilton (HH) stage 9. The optic vesicles constrict at their bases and the optic stalk is established at HH stages 11 and 12, respectively. At HH stage 14, the optic vesicle begins its invagination and the optic cup is formed by HH stage 15. By HH stage 20, pigmentation in the eye is evident. For our purposes, we are mainly interested in HH stage 11 and 18 embryos, as we have chosen these stages for our retroviral-mediated gene transfection studies.

1.3. *Previous experiments*

As already mentioned, earlier research performed by our lab, in particular by Dwayne Bisgrove, has demonstrated that AP-2 negatively regulates transcription of the retinal fatty acid binding protein (*R-FABP*) gene. FABPs are a family of related molecules that are involved in processes such as the uptake, storage, and solubilization of fatty acids. Chicken

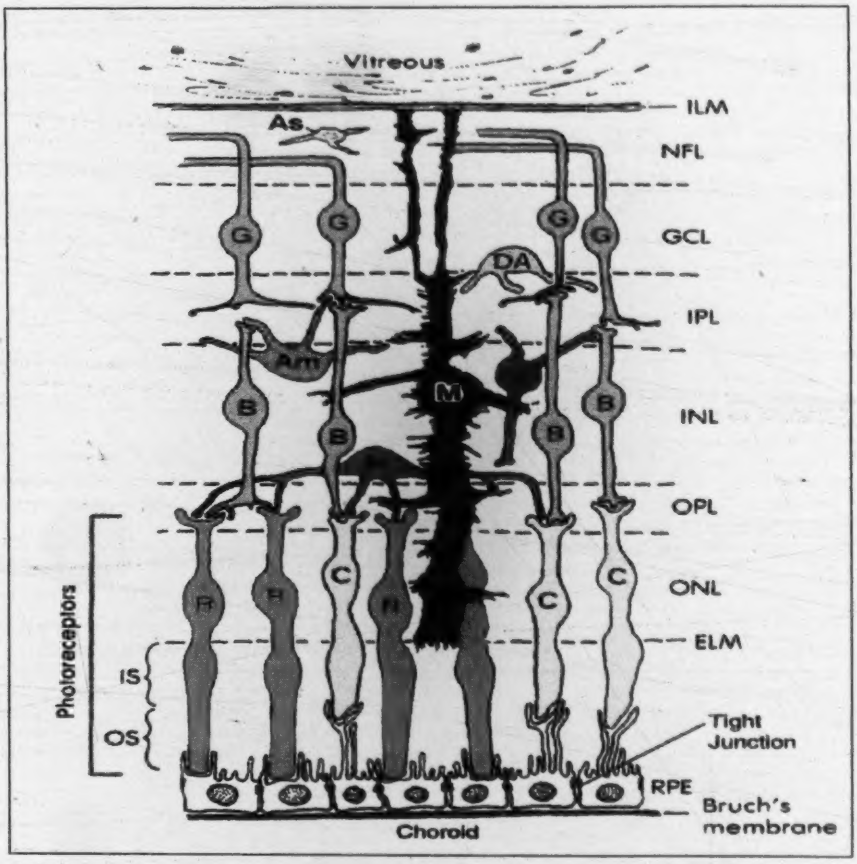

Figure 1.2.2: Organization of cells in the retina. The various cell types as well as the different retinal cell layers are shown in this diagram. G: ganglion cells; DA: displaced amacrine cells; Am: amacrine cells; I: interplexiform cells; M: Müller glial cells; B: bipolar cells; H: horizontal cells; R: rod photoreceptors; C: cone photoreceptors; ILM: inner limiting membrane; NFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; ELM: external limiting membrane; RPE: retinal pigmented epithelium (Figure adapted from Ogden, 1987).

R-FABP appears to be the avian equivalent of mammalian B-FABP. Analysis of the *R-FABP* promoter by DNA footprinting revealed four distinct protein binding sites, one of which contained the sequence 5'GCCGTGGGC3' that matched the AP-2 consensus site. EMSA analyses were subsequently performed on d7 and d16 chick retina nuclear extracts with a DNA fragment containing this site as the probe. A protein:DNA complex was observed which disappeared upon the addition of cold competitor AP-2 oligonucleotide to the reaction. Notably, when an anti-AP-2 antibody was introduced into the reaction, the complex supershifted, indicating that an AP-2-like protein bound to the DNA. CAT assays were performed with the reporter sequence fused to a fragment of the *R-FABP* promoter containing the AP-2 binding site as well as to constructs harbouring AP-2 sites that had been mutated. While transcriptional activity was observed from the *R-FABP* promoter, a decrease in activity was not observed from the mutant constructs, leading to the hypothesis that AP-2 was repressing transactivation from the *R-FABP* promoter in these experiments (Bisgrove *et al.*, 1997).

Further experiments have lent more support to this hypothesis. For instance, reporter assays were performed on d5 primary chick retinal cultures: a CAT construct with the *R-FABP* promoter was co-transfected with either an AP-2α or AP-2β expression vector. In both cases, activity from the reporter gene was significantly reduced when compared to the amount of activity observed in control transfections. *In situ* hybridizations also revealed mutually exclusive expression patterns for *AP-2* and *R-FABP* in the chick retina, suggesting that AP-2 may be repressing the expression of *R-FABP*. Specifically, at ED7 *R-FABP* was expressed throughout the retina while *AP-2α* and *–β* were just beginning to show expression in the inner nuclear layer. With differentiation, transcripts for both *AP-2* genes were found in the inner portion of the INL, the location of amacrine cells, while *R-FABP* expression was restricted to the outer half of the INL where the *AP-2* transcripts were absent. *AP-2β* transcripts were also observed in the horizontal cell layer and in displaced amacrine cells in these experiments. As well, Northern and Western blot analyses determined that AP-2 expression peaks in the retina at approximately ED10 (Bisgrove and Godbout, 1999).

1.4. *Thesis Objectives*

In light of the finding that AP-2 is expressed in the horizontal and amacrine cells of the developing chick retina, and the fact that this protein is known to regulate a vast number of genes, it is our hypothesis that additional target genes of AP-2 exist in these cells. We also contend that these putative target genes may play a key role in early retinal development and that their identification will advance our current understanding of retinal maturation during embryogenesis. Thus, we have attempted to isolate these novel target genes through a variety of methods including differential screening of a cDNA library, suppression subtractive hybridization, literature searches, and chromatin immunoprecipitation (ChIP).

In addition, we are interested in the effects that inappropriate AP-2 expression has on the differentiating retina. To this end, we have cloned the coding sequence for the *AP-2* gene into the RCAS and RCAN system of retroviral vectors (Hughes *et al.*, 1987). Using *in ovo* electroporation (for a review of this procedure see Ogura, 2002), we have attempted to deliver exogenous AP-2 into the developing chick retina to examine its effects. Specifically, we are trying to introduce premature AP-2 expression in the retina; we predict that this may have a significant impact on retinal cells, perhaps by forcing horizontal and amacrine cells to differentiate at an earlier stage than normal.

CHAPTER TWO

Materials and Methods

2.1. General Molecular Biology and Biochemical Techniques

2.1.1. *Plasmid DNA Preparation*

In all instances, plasmid DNA was prepared using a protocol similar to the lysis by boiling method for plasmid DNA preparation described by Sambrook *et al.* (1989). 5 ml aliquots of LB were inoculated with single bacterial colonies and grown overnight at 37°C with shaking. The cultures were centrifuged the following day at 2000 rpm for 10 minutes at 15°C, and the pellets were resuspended in 700 µl of buffer (8% sucrose, 0.5% Triton® X-100, 5 mM EDTA pH 7.5, 50 mM Tris-HCl pH 7.5). Freshly prepared lysozyme was added to a final concentration of 1.5 µg/µl; the cells were briefly vortexed and incubated for 5 minutes at room temperature. The samples were then boiled for 50 seconds and centrifuged for 10 minutes at 14 000 rpm at room temperature. The pellets were removed, and 700 µl of cold isopropanol was added. The tubes were inverted several times, placed at –80°C for at least 10 minutes, and spun for 15 minutes at 11 000 rpm at 4°C. The pellets were washed in 75% ethanol and resuspended in 100 µl of TE. 100 µl of 7.5 M NH_4oAc and 500 µl of 95% ethanol were then added to each of the tubes. The –80°C incubation, 4°C spin, and ethanol wash were repeated. The pellets were dried in a SpeedVac® (Savant Instruments Inc.) and resuspended in 75-80 µl of TE (10 mM Tris-HCl and 1 mM EDTA). All DNA was stored at –20°C.

2.1.2. *Restriction Endonuclease Digestions and Agarose Gel Electrophoresis*

Restriction endonuclease digestions of plasmid DNA generally contained ~1 µg of DNA, a 3-4 molar excess of an appropriate restriction enzyme(s), a suitable digestion buffer, and, if required, BSA in a total reaction volume of 20 µl. Reactions were incubated at 37°C for several hours to overnight[*]. 3 µl of loading dye (50% sucrose in TE with 0.3% BPB) was added to each digest prior to gel electrophoresis. If necessary, RNase A was also added to the sample prior to loading (final concentration of 50 ng/µl) to remove RNA. 1% agarose gels were made with Howley buffer (40 mM Tris, 33 mM NaoAc, 1 mM EDTA, adjusted to a pH of 7.2), electrophoresed for several hours, and stained with ethidium bromide before visualization on a UV light box. Photographs of gels were taken with either a Polaroid MP4 Land Camera or with an Alpha Imager™ 2000 (Alpha Innotech Corporation).

2.1.3. *DNA Sequencing and BLAST Searches*

DNA sequencing by capillary gel electrophoresis was carried out on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). The sequencing reactions (10 µl volume) contained ~500 ng of plasmid DNA, 1 µM of an appropriate primer, sterile H_2O, and 4 µl of BigDye™ terminator mix. The reactions took place in a thermal cycler (GeneAmp® PCR System 9700 or 2400 (Perkin Elmer)) and consisted of 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. The completed reaction products were precipitated with 2.5 volumes of 95% ethanol and a 1/10 volume of 3 M NaoAc (pH 5.2). The DNA was incubated for 15 minutes at room temperature and spun for 20 minutes at 14 000 rpm. The pellets were washed twice in 75% ethanol, dried in a SpeedVac®, resuspended in 12.5 µl formamide (Fluka), boiled for 5 minutes, and chilled on ice for 10 minutes. The samples were then loaded on the machine. Once DNA sequence was obtained with the ABI PRISM® 310 Collection Program (version 3.0.0), it was subjected to a BLAST analysis (www.ncbi.nlm.nih.gov/BLAST).

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[*] Digests that included *Sma* I were incubated at 25°C.

2.1.4. *Northern Blot Construction*

Extra precautions were taken in the construction of Northern blots, such as wearing gloves and using equipment dedicated to RNA work, to eliminate RNAse contamination. A 1.5% gel was prepared containing sterile H_2O, filtered MOPS buffer (10X stock: 0.2 M MOPS, 50 mM NaoAc, 10 mM EDTA, adjusted to a pH of 7.0), 6.3% formaldehyde (J. T. Baker), and agarose. Meanwhile, mRNA was added to a mix (comprised of 65% formamide, 8.4% formaldehyde, and 1.3X MOPS) and heat shocked. Three microlitres of loading buffer (1X MOPS, 50% glycerol, 0.1% BPB) was added and the samples were loaded onto the gel, which was run for 5-6 hours at 40 mA in 1X MOPS buffer. A nitrocellulose membrane was prepared by wetting it in sterile H_2O for 10 minutes; this was followed by several minutes of equilibration in 20X SSC. The RNA was allowed to transfer from the gel to the membrane by capillary action overnight in 20X SSC. The membrane was washed the following day in 2X SSC and air-dried between pieces of 3 MM Whatman® paper. The blot was baked in a vacuum oven at 80°C for two to 2.5 hours.

2.1.5. *Hybridization of Northern Blots, Probe Preparation, and Washes*

Northern blots were incubated with radioactively-labeled DNA probes in heat-sealed plastic bags. For blots ~60 cm^2 in size, 15 ml of pre-hybridization buffer was used (50% formamide, 5X SSC, 5X Denhardt's* solution, 50 mM $NaPO_4$ pH 6.5, and 0.25 mg/ml denatured salmon sperm DNA). The bag was immersed in a 42°C shaking water bath for a minimum of 4 hours.

The DNA (0.5 μg/6 μl) was labeled by nick translation. DNase I was diluted in activation mix (1 mg/ml BSA, 5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5) to create a working stock of 0.4 ng/μl. The following components were combined on ice in a final volume of 25 µl: NT buffer (5 mM Tris-HCl pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT, 50 µg/ml BSA), dGAT (C_f of 0.08 mM of each nucleotide), 0.5 µg of DNA, 7 µl of [α-^{32}P]dCTP (800 Ci/mmol, Amersham), DNase I (C_f of 0.016 ng/µl), and 7.5U of Kornberg DNA polymerase (Boehringer Mannheim). The reaction was placed in a 16°C H_2O bath for 35 minutes. After this incubation, 10 µl of stop buffer (125 mM EDTA, 10 mM Tris-HCl (pH 7.5), 0.1% SDS, 10 µg/ml single stranded DNA and 0.1% phenol red) was added to the tube, which was placed at 65°C for 10 minutes. The probe was then loaded onto a column filled with Bio-Gel® A-1.5m Bead Slurry (Bio-Rad) to separate the unincorporated nucleotides from the labeled probe by gravity. The collected probe was added to a 150-µl aliquot of salmon sperm DNA (10 mg/ml). The probe/salmon sperm DNA mixture was boiled for 10 minutes and chilled on ice. The denatured probe was then added to a tube containing hybridization mix (a mixture containing 50% formamide and 30% hybridization cocktail (15X SSC, 3X Denhardt's solution, 60 mM $NaPO_4$ pH 6.5, and 30% dextran sulfate)), inverted thoroughly, and added to the blot. The blot was incubated at 42°C overnight and washed the next day.

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* 100X Denhardt's solution contains 2 g of PVP, 2 g of BSA, and 2 g of Ficoll® 400 in 100 ml of H_2O.

The washes consisted of three five-minute low stringency washes with a solution containing 0.1% SDS and 2X SSC at room temperature on a shaker. This was followed by two high stringency washes with a solution containing 0.1% SDS and 0.1X SSC for one hour at an appropriate temperature. The temperature of the high stringency wash was dependent on the length of the probe (in general, 55°C was used for probes ~500 bp in length, 45-55°C was used for probes ~200-400 bp, and 58-60°C was used for fragments greater than 500 bp). The nitrocellulose blot was dried and exposed to film. Blots to be probed multiple times were stripped of probe by two washes in 0.05X Thomas buffer (10X Thomas buffer: 0.5 M

Tris-HCl pH 8, 20 mM EDTA, 5% sodium pyrophosphate, 0.2% BSA, 0.2% Ficoll®, and 0.2% PVP) for 1.5 hours each at 60°C.

2.1.6. *Nuclear Extract Preparation*

Nuclear extract from ED10 retinal tissue was prepared using the technique described by Dignam *et al.* (1983). Retinal tissue from two chick embryos, dissected in advance and stored at –80°C, was thawed on ice*. The cells were resuspended in a small amount of buffer A (10 mM HEPES-NaOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and freshly added 0.5 mM DTT and 0.5 mM PMSF.). Another small aliquot of buffer A was then added to the cells. This was followed by 10 minutes of incubation on ice and homogenization in a 1 ml dounce homogenizer with 10 strokes of a pestle. The mixture was spun at 1000 g for 10 minutes at 4°C. The supernatant was removed, and the remaining pellet (*i.e.* the crude nuclear extract) was centrifuged for an additional 20 minutes at 25 000 g to make it more compact. Following removal of the residual supernatant**, the pellet was resuspended in a small amount of buffer C (20 mM HEPES-NaOH pH 7.9, 25% glycerol, 0.42 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM DTT and 0.5 mM PMSF (freshly added)). The mixture was then homogenized as before, and centrifuged for 30 minutes at 25 000 g. The supernatant was transferred to a new tube, and a Bradford assay (Bradford, 1976) was performed to determine the yield of nuclear protein. Briefly, 800 µl of H_2O, 200 µl of Bradford reagent (Bio-Rad), and 1 µl of nuclear extract were combined, and read in a DU® 7400 spectrophotometer (Beckman) against a programmed standard curve. The extract was stored in aliquots at –20°C. ED7 nuclear extracts were also prepared in the same manner.

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[*] Typically, the yield of protein from this amount of tissue was ~500 µg. [**] If the cytosolic protein fraction was to be isolated, a 1/10 volume of buffer B (0.3mM HEPES-NaOH pH 7.9, 1.4 M KCl, and 30 mM $MgCl_2$) was added to the supernatant that was removed from the nuclear extract. This mixture was spun for one hour at 100 000 g and the supernatant was recovered for analysis.

2.1.7. *Whole Cell Extract Preparation*

Whole cell extracts were prepared from tissue culture cells that had been previously harvested and frozen (see below). A volume of Dignam buffer A (containing freshly added DTT and PMSF each to a C_f of 0.5 mM) approximately equal to the volume of the frozen cell pellet was added to the cells. A volume of a 50 mM Tris pH 8/1% SDS solution, also equal to the starting cell volume, was then added. The cells were taken up and down in a syringe with a 23-gauge needle until the solution reached a viscosity that could be pipetted. The Bradford assay (Bradford, 1976) was then performed to determine the protein yield.

2.1.8. *Western Immunoblot Construction, Immunoblotting, and Protein Detection*

SDS-polyacrylamide gels were prepared by first pouring a 15 ml separating gel (containing acrylamide:bis-acrylamide (30:0.8), separating buffer (375 mM Tris-HCl, 0.1% SDS, pH 8.8), 0.05% APS, and 7.5 µl TEMED) of an appropriate concentration. The separating gel was allowed to harden, and a 5.5% stacking gel was prepared and poured on top (30:0.8 acrylamide:bis-acrylamide, stacking buffer (125 mM Tris-HCl, 0.1% SDS pH 6.8), 75 µl APS, and 7.5 µl TEMED). An appropriate volume of loading dye (2X loading dye: 10% glycerol, 2% SDS, 0.5X stacking buffer, 0.003% BPB, and 40-50 mM DTT) was added to the samples, which were then boiled for ~3 minutes and loaded on the gel. 10 µl of broad range protein standards (Bio-Rad), also boiled, was included in one of the lanes. The gel was electrophoresed for ~50 min at 180V in Laemmli buffer (containing 25 mM Tris-HCl, 192 mM glycine, and 0.1% SDS, adjusted to a final pH of 8.3). The proteins were then transferred to a wetted nitrocellulose membrane for ~2.5 hours at 150 mA in transfer buffer (20% methanol, 25 mM Tris, and 192 mM glycine). The blot was placed in 0.005% CPTS (in 12 mM HCl) for several minutes to visualize the protein bands. It was then washed several times in TBS (150 mM NaCl, 50 mM Tris-HCl pH 7.5) and blocked in 10% milk

(Carnation™ milk powder in TBS) for 30 minutes on a platform shaker. The blot was washed twice in TBS for 10 minutes. An appropriate dilution of antibody in 5% BSA (in TBS) was added, and the blot was rotated in a sealed bag overnight at 4°C.

The next day, the blot was washed twice in TBS with 1% Tween-20, and once in TBS (each wash was 10 minutes in duration). A suitable secondary antibody at an appropriate concentration in 5% milk was added to the blot, which was shaken for ~4 hrs at room temperature. The TBS/1% Tween-20 and TBS washes were repeated, and ECL™ western blotting detection reagents (Amersham-Pharmacia) were applied to the blot for one minute. The ECL™ mixture was removed, and the blot was covered in SaranWrap® and exposed to X-ray film.

2.1.9. *DNA Ligations and Transformations*

DNA ligations were generally carried out in a total reaction volume of 20 μl. The following components were added to an Eppendorf tube: ligation buffer (C_f of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT), ATP (C_f of 1 mM), BSA (C_f of 0.1 μg/μl), an appropriate volume of digested vector DNA*, a suitable volume of insert DNA, and 1U of T4 DNA ligase (GIBCO BRL®). The reaction was incubated overnight at 16°C.

Transformations of the ligated DNA were performed the following day. An aliquot of suitable competent cells was thawed on ice. Half of the ligation reaction was diluted 1/5 with TE. If plasmid DNA was to be transformed, the DNA was diluted 1/5000. 100 μl of cells were added to the diluted DNA and gently vortexed. The tube was incubated on ice for 20 minutes and then placed at 37°C for 5 minutes. 5 ml of LB was added and the tube was shaken at 37°C for 30 minutes. The tube was then spun for 10 minutes at 2000 rpm at

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[*] If vector DNA was cut with a single enzyme, the ends were dephophorylated with calf intestinal alkaline phosphatase. 0.5 μl of enzyme was added to the digested DNA, which was then incubated for 45 minutes at 37°C. The DNA was purified by electroelution on a gel.

15°C. The cells were resuspended in the small amount of residual liquid remaining after decanting the supernatant and plated. If colour selection was required, 1% top agar was melted, IPTG and X-gal were added (to a C_f of 0.3 mg/ml each), and the solution was poured on the surface of a pre-warmed LB plate containing ampicillin. Plates with transformed cells were incubated overnight at 37°C.

2.1.10. *DNA Insert Purification by Electroelution*

DNA (generally 30 μg) was digested with an appropriate restriction endonuclease(s), loaded onto a 5% TBE/polyacrylamide gel, and electrophoresed for ~2.5 hours at a 150-170V in TBE buffer (4.5 mM TrisHCl, 3.5 mM boric acid, 4 mM EDTA, adjusted to a final pH of 8.1). The gel was stained with ethidium bromide, and the band(s) of interest was excised under UV light. The gel slice was placed in dialysis tubing (with a molecular weight cut off of 12-14 000 Da) filled with a small amount of TBE buffer to undergo electrophoresis in a gel box also filled with buffer. Following electroelution, TE was added to the bag to rinse it, and the contents were transferred to a polypropylene tube. A 1/10 volume of 5 M NaCl was added, and the DNA was extracted with equal volumes of organic solvents (*i.e.* 1X chloroform:isoamyl alcohol (24:1), 1X phenol, 1X chloroform:isoamyl alcohol (25:24:1), and 1X ether). 2.5 volumes of 95% ethanol were added, and the tube was incubated at –20°C overnight. The next day, the DNA was spun at 8000 rpm for 30 minutes at 4°C, and the pellet was washed in 75% ethanol. The supernatant was carefully removed, and the DNA was dried under vacuum. The dried pellet was resuspended in an appropriate volume of H_2O or TE. A few microlitres was set aside for agarose gel electrophoresis, and the sample was stored at –20°C.

2.1.11. *Dissection of Chick Retinae*

Instruments were rinsed in 75% ethanol prior to dissection of retinal tissue. With the aid of a dissecting microscope, sacrificed embryos were enucleated. The lens and vitreous humor were removed, exposing the underlying retina, which was separated from the rest of the tissue. The retinas were transferred in a small amount of PBS to Eppendorf tubes, flash frozen in liquid nitrogen, and stored at –80°C.

2.1.12. *Establishment of d5 Primary Retinal Cultures, Transfections, and Harvesting*

Retinae from two chicks (*i.e.* four eyes) were collected for each 100 mm plate to be established. The tissue was pipetted to break up clumps and plated in DMEM medium (GIBCO BRL®) containing 10% FCS and penicillin/streptomycin. The plates were incubated at 37°C with 5% CO_2. The cells were cultured for four days and then transfected using the calcium phosphate-mediated DNA transfection method (Kingston, 1996). Briefly, 10 μg of plasmid DNA was mixed with 250 mM $CaCl_2$ in a total volume of 500 μl for each 100 mm plate. The tube was vortexed twice. A one ml glass pipet attached to a Peristaltic Pump P-1 (Pharmacia) was placed into a second tube containing 0.5 ml of HEPES solution (0.595 g HEPES, 0.818 g NaCl, 0.009 g NaH_2PO_4 per 50 ml, with a pH of exactly 7.12). Bubbles were allowed to form at a slow rate in the HEPES solution, while the DNA/$CaCl_2$ solution was added drop-wise. The tubes were incubated at room temperature for 30 minutes to allow precipitate formation. The mixture was then added evenly to each plate of cells. The next day, the cells were washed in PBS, and fresh medium was added. The cells were returned to the incubator and harvested several days post-transfection.

To prepare whole cell extracts from transfected cells, plates were washed twice with 10 ml of PBS. A small amount of fresh PBS was added to the plate, and the cells were scraped with a rubber policeman. The cells were then spun for 7 minutes at 1500 rpm. The supernatant was removed, and the cell pellets were stored frozen at –80°C. If the cells were ultimately destined for RNA extraction, they were harvested by trypsinization (with 1.5 ml of 0.25% trypsin in EDTA/PBS and a five-minute incubation at 37°C) rather than scraping.

2.1.13. *RNA Preparation from Transfected Cells Post-Harvesting*

Frozen cell pellets were resuspended in 0.5 ml of 50 mM NaoAc pH 5.2 and vortexed. 6 ml of 50 mM NaoAc/1% SDS and 6 ml of 60°C phenol, equilibrated with 50 mM NaoAc pH 5.2, were then added to the tube, which was vortexed following each addition. The tube was incubated in a 60°C water bath for 15 minutes with frequent inversion, and placed in a salt/ice water bath for 5 minutes. The tube was spun at 3000 rpm for 10 minutes at 4°C, and the phenol was removed, leaving the interphase behind. A second phenol extraction was performed. This was followed by the addition of chloroform:isoamyl alcohol (24:1), and the tube was mixed for 10 minutes. The tube was spun as before, and the upper phase was transferred to a new tube. A 1/10 volume of 5 M NaCl and two volumes of 95% ethanol were added, and the tube was placed at –20°C overnight. The sample was spun at 3000 rpm for 50 minutes at 4°C. The supernatant was carefully decanted, and the pellet was dried under vacuum. The dried RNA pellet was resuspended in 25 μl of TE and stored at –80°C. If poly(A)+ RNA was desired, it was prepared by running the total RNA through oligo (dT) cellulose columns (Dr. Roseline Godbout prepared the mRNA used in the construction of Northern blots.).

2.2. Differential Screening of a cDNA Library to Identify Genes Regulated by AP-2

mRNA obtained from ED7 chick retinae was used to construct a cDNA library in Lambda ZAP bacteriophage (Stratagene). Plates containing infected cells were generated and plaque lifts were performed in duplicate. One filter was incubated with ^{32}P-labelled cDNA probe from d5 primary retinal cultures transfected with an AP-2β sense construct in pcDNA3. The other filter was probed with cDNA obtained from cultures transfected with the corresponding antisense AP-2β construct. After washing and exposing the filters to film, plaques showing differences in signal intensity between the two filters were picked for secondary and tertiary rounds of screening. The genetic material from positive plaques, containing insert DNA from the library, was converted to plasmid (*i.e.* pBluescript®) form. Dr. Roseline Godbout constructed the library and performed the screens, while Dwayne Bisgrove generated the AP-2β constructs. DNA from these plasmids was obtained for further analyses.

2.3. Suppression Subtractive Hybridization (SSH)

SSH was carried out using the PCR-Select™ cDNA Subtraction protocol (Clontech Cat. #:K1804-1). Figure 3.2.1 contains a diagram that outlines this technique.

2.3.1. *cDNA Preparation*

Poly A+ RNA obtained from the d5 primary retina cultures that had been transiently transfected with the sense AP-2β expression construct (see section 2.2) was the tester population in the SSH procedure. Conversely, the driver population consisted of mRNA from the d5 retina cultures transfected with the antisense AP-2β construct. cDNA was synthesized from the two mRNA populations: 2 μg of mRNA was combined with cDNA synthesis primer (C_f of 1 μM) in a 5-μl volume. The reactions were incubated at 70°C for 2 minutes and cooled on ice for 2 minutes. First strand buffer (C_f of 50 mM Tris-HCl (pH 8.5), 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT), dNTPs (C_f of 1 mM each of dATP, dCTP, dGTP, dTTP), and 20U of AMV reverse transcriptase were added to the tubes (10 μl total volume), which were then incubated at 42°C for 1.5 hours. The tubes were placed on ice, and second strand buffer (C_f of 100 mM KCl, 10 mM ammonium sulfate, 5 mM $MgCl_2$, 0.15 mM β-NAD, 20 mM Tris-HCl (pH 7.5), 0.05 mg/ml BSA), dNTPs (C_f of 0.2 mM), and second strand enzyme cocktail (containing DNA polymerase I, RNAse H, and *E. coli* DNA ligase) were added (V_f of 80 μl). The reaction tubes underwent two hours of incubation at 16°C. Then, 6U of T4 DNA polymerase was added, and the reactions were incubated for an additional 30 minutes at 16°C.

EDTA/glycogen mix (C_f of 10 mM EDTA; 0.05 mg/ml glycogen) was added to stop the reactions, which were then extracted with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1), chloroform:isoamyl alcohol (24:1), and ether. A 1/10 volume of 4 M NH_4oAc and 2.5 volumes of 95% ethanol were added to the samples, which were centrifuged at 14 000 rpm for 20 minutes at 4°C. The supernatant was removed, and the pellet was washed with 80% ethanol, dried, and resuspended in H_2O. Each population then was subjected to *Rsa* I digestion for ~1.5 hours at 37°C. As before, EDTA/glycogen mix was added, and the samples were extracted with organic solvents. NH_4oAc and 95% ethanol were added to the tubes, which were centrifuged again. The pellets were washed with 80% ethanol and dried. The pellets were then dissolved in 5.5 μl H_2O and stored at –20°C.

2.3.2. *Ligation of Adaptors to Tester DNA*

The tester and driver cDNA isolated above (in 5.5 μl) were diluted 1/6 with H_2O. Two ligations were then set up with *only* the tester DNA: the first ligation included Adaptor 1 and the second ligation was done with Adaptor 2R (2 μl diluted cDNA, adaptor 1 *or* 2R (C_f of 2 mM), ligation buffer (C_f of 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 2 mM DTT, 0.05 mg/ml BSA), and 400U T4 DNA ligase (containing 3 mM ATP)). After an overnight incubation at 16°C, EDTA/glycogen was added to the tubes, which were then heated for 5 minutes at 72°C. The remaining 2 μl of diluted tester DNA not used in the ligations was mixed with 2 μl of driver cDNA to become an unsubtracted control for PCR.

2.3.3. *Hybridization*

In two separate PCR tubes, 1.5 μl of *Rsa* I digested driver cDNA, 1.5 μl of the Adaptor 1 *or* Adaptor 2R ligation, and the hybridization mix (50 mM HEPES pH 8.3, 0.5 M NaCl, 0.02 mM EDTA pH 8.0, 10% PEG 8000) were combined. The tubes were incubated in a thermal cycler for 1.5 minutes at 98°C and then for 8 hours at 68°C. 1 μl of driver cDNA (with *no* adaptors) was combined with the hybridization mix (V_f of 4 μl). 1 μl of this mixture was placed in a PCR tube, incubated at 98°C for 1.5 minutes, and all three samples (*i.e.* the Adaptor 1 hybridization, the Adaptor 2R hybridization, and the fresh denatured driver) were combined. The entire reaction was pipetted up and down to mix and incubated overnight at 68°C.

2.3.4. *Amplification, Purification, and Cloning of Subtracted cDNA*

PCR of the unsubtracted tester control (section 2.3.2) was performed to test the ligation efficiency. A hot start PCR was set up by combining H_2O, Stratagene PCR buffer (10X: 1 M NaCl, 100 mM Tris-HCl (pH 8.5), 100 mM $MgCl_2$, 10 mM DTT, 100 μg/ml BSA), 0.2 mM dNTPs, 0.4 μM of primer 1, and 1 μl of template (V_f of 25 μl). The tube was incubated for one minute at 75°C. 0.75 μl of *Taq* DNA polymerase was added, and the reaction was incubated for 5 more minutes at 75°C to fill in the ends of the adaptors. 30 cycles of 94°C for 10 seconds, 66°C for 30 seconds, and 72°C for 1 minute and 30 seconds were performed. A secondary PCR was set up which consisted of Platinum *Taq* High Fidelity buffer (GIBCO BRL®, 10X: 600 mM Tris-SO_4 (pH 8.9), 180 mM $(NH_4)_2SO_4$), 2 mM $MgSO_4$, 0.4 μM of primer 1[*], 0.2 mM dNTPs, 1 μl of a 1/10 dilution of the first reaction, and 2.5U of Platinum *Taq* High Fidelity DNA polymerase (GIBCO BRL®). The PCR products were analyzed on an agarose gel, following 15 cycles of 94°C for 10 seconds, 65°C for 30 seconds, and 72°C for 1 minute 30 seconds.

Once the ligation efficiency was tested, a PCR reaction of the subtracted d5 retina cDNA was set up. The reaction conditions were the same as those just described except that 1 μl of the subtracted d5 retina cDNA was placed into the primary reaction. A secondary PCR was then performed with 1 μl of a 1/10 dilution of the first reaction as the template. A 5-μl aliquot of the final PCR products was loaded on an agarose gel for visualization. The reaction was repeated in triplicate, and the products were combined and prepared for cloning.

The Concert™ Rapid PCR Purification System (GIBCO BRL®) was used to purify the amplified subtracted cDNA according to the manufacturer's directions. This system involves the use of a spin cartridge containing a silica-based matrix. The DNA binds to this matrix, while primers and unincorporated nucleotides flow through. After binding the DNA to the column and washing it, the DNA was eluted from the matrix by adding 50 μl of 65°C H_2O to the column and collecting the flow through of a subsequent centrifugation. The recovered DNA was digested sequentially with the restriction endonucleases *Sma* I and *Eag* I; the salt concentration was adjusted before the addition of the second enzyme to create optimal cutting conditions for both. The digested insert DNA was purified with the Concert™ system as before. The recovered DNA was precipitated overnight in the presence of a 1/10 volume of 5 M NaCl and 2.5 volumes ethanol. The final product was resuspended in 10 µl of H_2O. The pBluescript® vector (Stratagene) was digested with *Eag* I and *Eco*R V to generate cohesive ends for cloning. The cut vector was electroeluted and purified as usual. Ligations between the insert DNA fragments and the digested plasmid were set up in the usual manner. XL1Blue cells were transformed and plated on prepared X-gal/IPTG LB-Amp plates. White colonies were picked, and plasmid DNA from the SSH-clones was obtained for further investigations.

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[*] One of the primers in the nested primer set recommended by Clontech had an unusually high Tm. Amplification using these primers under the specified conditions proved unsuccessful; thus, the secondary reaction was performed again with primer 1.

2.3.5. *Probe Preparation for Slot Blotting Analysis of SSH Clones*

cDNA, for radio-labeling and probing of slot blots, was synthesized from the mRNA obtained from d5 primary retinal cell cultures transfected with either the AP-2β sense or the AP-2β antisense pcDNA3 expression construct (see section 2.2). 2 µg of mRNA was combined with 1 µg of oligo-dT primer in a total volume of 10 µl. The reaction was heated at 70°C for 10 minutes and chilled on ice for five. Then, first strand synthesis buffer (5X: 250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 15 mM $MgCl_2$ from Superscript RT kit (GIBCO BRL®)), 34U of RNase guard, sodium pyrophosphate (C_f of 3 mM), dGATC (C_f of 0.7 mM), DTT (C_f of 13 mM), spermidine (C_f of 0.3 mM), and 200U of Superscript reverse transcriptase (GIBCO BRL®) were added (V_f of 30 µl). The reaction was placed at 42°C for 1.5 hours. Synthesis of second strand cDNA proceeded by adding second strand synthesis buffer (C_f: 40 mM Tris-HCl pH 7, 90 mM KCl, 3 mM $MgCl_2$, 3 mM DTT, and 0.05 µg/µl BSA), 10U of Klenow DNA polymerase (Boehringer Mannheim), several units of RNAse H, and H_2O to the first strand reaction (V_f of 125 µl). The reaction was placed at 16°C for 2 hours and then heated at 70°C for 10 minutes. 2U of T4 DNA polymerase (Roche) was added, and the tube was incubated for 10 minutes at 37°C. The reaction was stopped with EDTA (C_f of 20 mM). Each synthesis reaction was then extracted with organic solvents and precipitated with a 1/10 volume of 3 M NaoAc pH 5.2 and two volumes of 95% ethanol and the final dried pellets were resuspended in H_2O.

The probes were radioactively labeled by the random priming method. 50 ng of DNA in 70 μl of H_2O was boiled for several minutes and equilibrated at 37°C. 12 μl of OLB (5X OLB: 1 M HEPES pH 6.6, hexadeoxyribonucleotides (27 OD_{260}/ml), 0.1 mM dATP, dGTP, dTTP, 50 mM β-mercaptoethanol, 0.25 M Tris-Cl, pH 8.0, 25 mM $MgCl_2$), 10 μl of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham), and 8U Klenow DNA polymerase (Amersham Pharmacia) were added, and the reaction was incubated for 2 hours at 37°C. NT stop buffer was added to the tube, which was then incubated for 10 minutes at 65°C. The DNA was purified on a column and prepared for hybridization to a slot blot in the same manner as for northern blotting.

2.3.6. *Slot Blotting Analysis of Clones Obtained from SSH*

To generate sufficient DNA for analysis, several identical mini-plasmid DNA preparations from each SSH clone were combined and digested with restriction enzymes to liberate the inserts. Slot blots were constructed with this DNA, following its electroelution and purification, according to a protocol outlined by Brown (1993). A positively charged nylon membrane was cut to fit a Schleicher and Schuell Minifold II apparatus and soaked in 6X SSC for 10 minutes. The membrane was laid inside the manifold on top of two Whatman® 3MM filter papers, also wetted in 6X SSC. The samples were then prepared for loading: 8 μl of insert DNA, SSC (C_f of 6X), and H_2O (to bring the volume up to 425 μl) were combined. The samples were denatured by boiling for 10 minutes and placed on ice. In the meantime, suction was applied to the manifold, and 500 μl of 6X SSC was loaded into each well. 200 μl of each sample was added to a well in the top half of the apparatus, while 200 μl was added to a corresponding well in the bottom half. Thus, by cutting the blot in half, duplicate blots containing equivalent amounts of DNA were made. The membrane was placed DNA side up on a piece of Whatman® 3MM filter paper soaked in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 10 minutes. Then, the membrane was neutralized with a 1 M NaCl/0.5 M Tris-HCl pH 7 solution for five minutes. The membrane was air-dried on a fresh piece of filter paper and baked for 1 hour and 30 minutes at 80°C in a vacuum oven. This was followed by UV cross-linking in a UV Stratalinker™ 2400 (Stratagene). Each blot was hybridized to one of the probes (section 2.3.5), washed, and exposed to X-ray film. Clones showing a difference in expression between the two blots were selected for further analysis.

2.4. RT-PCR of Putative AP-2 Target Genes Identified from the Literature

2.4.1. *First Strand cDNA Synthesis for RT-PCR*

mRNA from ED10 and ED16 chick retinae was used for RT-PCR. The first strand cDNA synthesis reaction consisted of 1 μg of mRNA, 5 mM of oligo-dT primer ($T_{12}MN$), dNTPs (C_f of 0.5 mM), 27U of RNAse inhibitor, spermidine (C_f of 0.5 mM), DTT (C_f of 10 mM, and first strand buffer (GIBCO BRL®, see above), in a reaction volume of 20 μl. The reaction was incubated at 65°C for 5 minutes and then chilled on ice. 200U of Superscript reverse transcriptase was added; the reaction was incubated at 42°C for one hour. The tube was then heated at 95°C for 5 minutes and stored at –80°C.

2.4.2. *Primer Sequences and Reaction Conditions*

The PCR primers 5'CAACTGCTACATGGAGGAGGC3' (sense) and 5'GAATCCGACAGAGCTGGCTTG3' (antisense) were used for isolating an approximately 520 bp fragment from the coding region of the chicken choline acetyltransferase gene (*ChAT*, GI: 15341175). The amplification reaction (50 μl) contained

Stratagene PCR buffer (see above), 0.2 μM of each primer, 0.2 mM dGATC, 1 μl of first strand cDNA (from ED10 chick retina), and 1 μl of *Taq* DNA polymerase. The reaction took place in a GeneAmp® PCR System 9700 or 2400 (Perkin Elmer) programmed for the following: 94°C for 5 minutes (94°C for 1 minute, 64°C for 1 minute, 72°C for 1 minute) x 35 cycles, then 72°C for 7 minutes. The product was run on a 5% TBE polyacrylamide gel, the band was excised, and the DNA was eluted from the gel slice by placing it overnight at 37°C in 125 μl of TE. A secondary PCR was performed in triplicate using the same conditions as the first PCR reaction but with one μl of the eluted DNA as the template. The reactions were again run out on a gel, the bands were excised and electroeluted in TBE, and the DNA was extracted and precipitated as usual.

The primers used to isolate an approximately 460 bp cDNA fragment from the chicken dopamine β-hydroxylase gene (*DBH*, GI: 6983691) were 5'ATGAAGCCCTAGTCCACCAC3' (sense) and 5'TGGGAGGCGAAGATGCGGA3' (antisense). The reaction was like that described above for *ChAT*, except 1 μl of ED16 chick retina first strand cDNA was used. The conditions for the reaction were 94°C for 5 minutes (94°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute) x 35 cycles with a final extension at 72°C for 7 minutes. A secondary PCR (40 cycles of the same conditions) was performed with 1 μl of template eluted from a gel slice. The Concert™ Rapid PCR Purification System was used to purify the final PCR product according to the manufacturer's directions.

Finally, an ~900 bp cDNA fragment from the chicken tyrosine hydroxylase gene (*TH*, GI: 6523292) was first amplified with the sense primer 5'CCATCATGTCTCCACGGTTC3' and the antisense primer 5'GCCTCTTGATATGTGCTGCG3'. The conditions were as follows: 94°C for 5 minutes (94°C for 1 minute, 60°C for 1 minute and 12 seconds, 72°C for one minute) x 35 cycles and a 7-minute incubation at 72°C. ED16 chick retina first strand cDNA was used as the template. This reaction was followed by a nested PCR (with 5'AGAAGGACGGGA-GAGCCAT 3' (sense) and 5'CCATAGGAAGACAGGAGCC3' (antisense); 1 μl from the first reaction was used as the template. The cycling conditions were 94°C for 5 minutes (94°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute) x 35 cycles and 72°C for 7 minutes. The product was electroeluted and purified in the same manner as described for *ChAT*.

2.4.3. *Cloning of RT-PCR Products*

The TOPO-TA Cloning® System (Invitrogen) was used to clone the three RT-PCR products into the pCR®II-TOPO® vector for further manipulations. The procedure was followed as outlined by Invitrogen, with the exception that it was halved. 1 μl of each PCR product was used in the cloning reaction as directed. Then, 25 μl of competent TOP10® *E. coli* cells (Invitrogen, $1X10^9$ cfu/μg supercoiled DNA and *lacZΔM15*) were transformed with 2 μl of this reaction. The cells were plated on LB-Amp plates containing X-gal and IPTG. Plasmid DNA was recovered, digested to confirm the presence of an insert, and sequenced. Once the identity of the DNA was confirmed, insert DNA was obtained via electroelution for labeling and hybridization to Northern blots.

2.5. Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was carried out following several published protocols (Weinmann *et al.*, 2001; Weinmann and Farnham, 2002; web page of Dr. P. J. Farnham, http://mcardle.oncology.wisc.edu/farnham/protocols/chips.html; Aparicio, 1999; Kuo and Allis, 1999; Orlando *et al.*, 1997; Upstate Biotechnology chromatin immunoprecipitation protocol, www.upstatebiotech.com) with a number of modifications.

2.5.1. *Tissue Cross-linking*

Retinae from ED10 chick embryos* were dissected in PBS and minced with a sterile razor blade or forceps. PBS was added to the tissue to bring the volume to 5 ml. Formaldehyde (37% stock, J. T. Baker) was added to a final concentration of 1% and the tube was rotated for five minutes on a nutator at room temperature (the optimization of cross-linking is discussed in section 2.5.2). Glycine was added to a final concentration of 0.125 M to quench the reaction; the tube was rotated for an additional five minutes. The tissue was spun down for several minutes at 1500 rpm at 4°C and washed in PBS. The tissue was resuspended in 1 ml of PBS, transferred to an Eppendorf tube, and flash frozen in liquid nitrogen for storage at –80°C.

2.5.2. *Optimization of Cross-linking of ED10 Retinal Tissue and Sonication*

Tissues were treated with various concentrations of formaldehyde for different amounts of time, washed in PBS, and resuspended in nuclear lysis buffer (63 mM HEPES pH 7.6, 3% sarkosyl, 6.25 mM EDTA, and 3 mM EGTA) with Complete™ protease inhibitors (Roche). The samples were sonicated, spun at 14 000 rpm for 10 minutes at 4°C to remove debris, and loaded on a CsCl gradient (from bottom to top, 1.11 ml of 1.75 g/ml CsCl, 0.77 ml of 1.5 g/ml, and 0.6 ml of 1.3 g/ml). The tubes were spun in an ultracentrifuge at 30 000 rpm for 20 hours at 20°C. Fractions of ~400 μl were collected by punching a hole in the bottom of the tube and allowing the samples to drip through. 20 μl from each fraction was run on a 1% agarose gel to determine the extent of cross-linking by examining the amount of free DNA remaining. Protocols for testing of cross-linking conditions were kindly provided by Dr. Charlotte Spencer.

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*A single embryo at this stage was determined to yield approximately 0.07 g of retinal tissue.

Suitable conditions for the DNA sonication step were also empirically determined. ChIP reactions were set up as in sections 2.5.1 and 2.5.4 (below) under various sonication conditions. The IP step (2.5.5) was omitted, and the procedure was resumed with the addition of 5 M NaCl to the samples (2.5.6). After resuspension of the final chromatin samples in H_2O and spectrophotometry to estimate the amount of DNA in the samples, several micrograms of DNA were run on an agarose gel to examine the size distribution of the DNA fragments. For cloning purposes, DNA fragments ranging in size from 500 bp to 1 kb were optimum.

2.5.3. *Bead Preparation for ChIP*

An appropriate volume (*i.e.* 25 µl per IP plus extra for preclearing) of Protein A Sepharose beads (Sigma) was washed at least three times in five volumes of ChIP dilution buffer (1% Triton® X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1). An equal volume of bead blocking solution (400 µg salmon sperm DNA, 1% BSA, 10 mM Tris-HCl pH 8.1, 1 mM EDTA) was then added to the packed bead volume to create a slurry, and the tube was rotated at 4°C for at least two hours. The blocked beads were stored at 4°C until needed.

2.5.4. *Chromatin Preparation*

Six tubes of cross-linked tissue were used to obtain enough material for cloning; the products were pooled following immunoprecipitation. Cross-linked tissue was thawed on ice and homogenized in a 1 ml glass dounce homogenizer with ~20 strokes of a loose pestle to break up the tissue. The homogenates were spun for several minutes at 1500 rpm at 4°C. The pellets were resuspended in 1 ml of cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40®) containing freshly added protease inhibitors (C_f of 1 mM PMSF, aprotinin to 0.01 mg/ml, and leupeptin to 0.01 mg/ml; or a working concentration of a dissolved

Complete^{TM} protease inhibitor tablet (Roche)). Another homogenization was performed (10 strokes with a tight pestle) followed by 10 to 15 minutes of incubation on ice. The samples were then spun in a microcentrifuge at 5000 rpm for 5 minutes at 4°C to pellet the nuclei. The resulting pellets were resuspended in 1 ml of nuclear lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS) containing protease inhibitors. The solutions were drawn up through a 23-gauge needle approximately 10 times to shear the DNA and left on ice for 20 minutes. The samples were kept on ice for sonication, which was performed with a Sonifier® Cell Disruptor 200 (Branson) with a 2 mm microtip (set to 50 percent duty in continuous mode on setting 6). Sonication consisted of a 30 second burst followed by two 15-second bursts. The samples were allowed to cool between intervals. The samples were then spun for 10 minutes at 14 000 rpm at 4°C to pellet the remaining cell debris. The supernatants were removed to new tubes for further manipulations.

2.5.5. *Immunoprecipitations*

300-500 μl aliquots of ChIP supernatant were added to Eppendorf tubes. The volumes were brought up to one ml with the addition of ChIP dilution buffer, containing protease inhibitors. The samples were then preblocked with 50 μl of prepared bead slurry and rocked for 30 minutes at 4°C. The tubes were centrifuged for two minutes at 14 000 rpm in a 4°C microcentrifuge. The supernatants were transferred to new tubes, 5 μl of anti-AP-2 antibody [anti-AP-2α C-18, lot A247 rabbit polyclonal antibody (100 μg/ml) *or* anti-AP-2α H-79, lot L061 rabbit polyclonal antibody at 200 μg/ml (Santa Cruz Biotechnology)] was added, and the samples were rocked overnight at 4°C. The following day, 50 μl of prepared bead slurry was added to each reaction and rotated for at least another 3 hours at 4°C. The tubes were spun at 14 000 rpm in a microcentrifuge for two minutes and the supernatants, containing protein not bound to the beads, were saved for western blot analysis.

A series of washes was then performed as follows: one wash with TSE I (0.1% SDS, 1% Triton® X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl), four washes with TSE II (0.1% SDS, 1% Triton® X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl), one wash with buffer III (0.25 M LiCl, 1% NP-40®, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1), and 2 washes in TE. One tube was set aside following the washes for western immunoblotting as a positive control for proteins obtained through ChIP.

2.5.6. *DNA Isolation*

150-200 µl of elution buffer (1% SDS, 100 mM $NaHCO_3$) was added to the beads, and the samples were vortexed on a platform for 15 minutes at room temperature. The tubes were spun for two minutes at 14 000 rpm in a microcentrifuge and the supernatants were collected. The elution step was repeated with another aliquot of buffer, and the products were combined. NaCl was added to a final concentration of 0.3 M, and the tubes were incubated at 65°C for 4-5 hours. Following this incubation, 2.5 volumes of 95% ethanol and 1 µl of yeast tRNA (10 mg/ml) were added, and the tubes were placed overnight at –20°C. The next day, the DNA was spun for 30 minutes at 4°C, washed with 75% ethanol, dried, and resuspended in 320 µl of TE. PK buffer (10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.25% SDS) was added along with 300 µg proteinase K. The samples were then incubated at 45°C for 1.5 to 2 hours. The samples were extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), the aqueous phases were removed, and the remaining organic phases were back extracted with equal volumes of TE. The second aqueous phase was combined with the first, and the pooled solutions were extracted a second time with phenol:chloroform. This was followed by a chloroform:isoamyl alcohol (24:1) extraction and the addition of a 1/10 volume of 5 M NaCl, 10 μg yeast tRNA, and 2.5 volumes of 95% ethanol to the aqueous solution. The samples were precipitated overnight at –20°C and spun the next day for 20 minutes at 14 000 rpm in a 4°C microcentrifuge. The pellets were washed in 75% ethanol and dried in a SpeedVac®. The individual pellets were resuspended and combined together in 50 μl of H_2O.

2.5.7. *Preparation of DNA Fragments for Cloning*

To clone the DNA products isolated from ChIP, the 5' and 3' ends of the DNA were blunted by T4 DNA polymerase. The reaction (in a 200 μl volume) contained the following components: the DNA recovered from ChIP, dNTPs (C_f of 0.1 mM for each nucleotide), 1X BSA (NEB), T4 DNA polymerase buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT), and 6U of T4 DNA polymerase (Roche). The reaction was incubated for 30 minutes at 37°C and then for 10 minutes at 70°C to inactivate the enzyme. The reaction was extracted with equal volumes of organic solvents (phenol:chloroform, chloroform, and ether). A one-tenth volume of 3 M NaoAc (pH 5.2) and 2.5 volumes of 95% ethanol were added, and the DNA was incubated overnight at –20°C. The DNA was spun at 14 000 rpm for 20 minutes at 4°C, washed in 80% ethanol, dried, and resuspended in a total volume of 12 μl H_2O.

2.5.8. *Linker Preparation*

*Eco*R I linkers [d(CCGGAATTCCGG), NEB] were phosphorylated with [γ-^{32}P]ATP to prepare them for ligation to the ChIP DNA. The 30 μl reaction consisted of kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.05 mM cold ATP, 0.1 mg/ml BSA), ~1 μg of the linkers, 2 μl of 10 μCi [γ^{32}P]ATP (Amersham), and 20U of polynucleotide kinase (NEB). The reaction was incubated for one hour at 37°C and terminated by the addition of EDTA (C_f of 20 mM). The reaction was then extracted with organic solvents. A 1/10 volume of 3 M NaoAc pH 5.2, 2.5 volumes of 95% ethanol, and 10 µg yeast tRNA were added, and the reaction was placed at –80°C for several hours. The phosphorylated linkers were spun for 30 minutes at 14 000 rpm at 4°C, washed in 80% ethanol, dried, and resuspended in 25 µl of H_2O.

2.5.9. *Linker Ligation to ChIP DNA*

5 µl of the labeled *Eco*R I linkers was ligated to the blunt-ended ChIP DNA (12 µl) in a volume of 70 µl, which also contained 3.5U of T4 DNA ligase. The following day, the reaction was extracted, precipitated, and resuspended in 40 µl of H_2O. 4 µl (10%) was removed at this point for gel analysis. The ChIP DNA was digested with *Eco*R I overnight at 37°C, and 5 µl (10%) of the digestion was removed for gel analysis. The two reserved aliquots, as well as the 45 µl of digested ChIP DNA and a molecular weight marker, were run on a 10% polyacrylamide gel. The portion of the gel containing the ladder and the bulk of the digest was stained with ethidium bromide, visualized under UV light, and fragments >300 bp were excised. The other half of the gel was dried and exposed to film. The excised gel slice, containing the ChIP DNA fragments, was electroeluted for several hours in TBE. The DNA was extracted and precipitated with 2.5 volumes of ethanol, a 1/10 volume of 5 M NaCl, and 10 µg yeast tRNA. The DNA was left overnight at –20°C, spun, washed, dried, and the final product was resuspended in 8 µl H_2O. Ligation reactions were set up with pBluescript® (Stratagene) cut with *Eco*R I and dephosphorylated by CIAP and the ChIP DNA with *Eco*R I ends. The reactions were incubated overnight at 16°C and the following day, TOP10® *E. coli* cells were transformed with 4 µl of each ligation reaction. The transformations consisted of 4 µl of the ligation mix and 50 µl of cells and proceeded according to the manufacturer's instructions (*i.e.* a 30 minute incubation on ice, 42°C heat

shock for 30 seconds, addition of growth medium, and one hour incubation at 37°C). The cells were spread on an LB-Amp plate prepared for colour selection. The resulting white colonies, containing possible inserts, were used to inoculate 5 ml of LB broth for plasmid DNA preparation.

2.5.10. *Analysis of Clones Isolated from ChIP*

The plasmid DNA isolated above was cut with the *Pvu* II restriction endonuclease to liberate a fragment containing the insert of interest. Clones with inserts estimated to be between 250 bp and 500 bp in length were selected for sequencing with either the T7 or T3 primers (5'AATACGACTCACTATAG3' and 5'ATTAACCCTCACTAAAG3', respectively). BLAST analyses were performed to provide possible identities for the DNA fragments. Sequences were also entered in DNA analysis programs and searches of promoter databases (www.ifti.org (MIRAGE; tfsitescan), www.gene-regulation.com (TRANSFAC; AliBaba2.1 and Patch), and Gene Tool (www.biotools.com)) were carried out to identify putative AP-2 binding sites. For clones that contained possible binding sites, DNA inserts were isolated from several identical combined plasmid DNA preparations by electroelution following digestion with either *Eco*R I *or Bam*H I and *Hin*d III. The concentration of the DNA was estimated by spectrophotometry using a DU® 7400 spectrophotometer (Beckman).

2.5.11. *Electrophoretic mobility shift assay (EMSA)*

EMSAs were performed using the insert DNAs from the clones isolated from ChIP as probes. 4 pmol of DNA* was used in each 20 µl labeling reaction. NT buffer (5 mM Tris-HCl pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT, 0.05 µg/µl BSA), an appropriate volume of DNA, dGCT (C_f of 0.25 mM of each nucleotide), 7 µl of [α-^{32}P]dATP (800 Ci/mmol,

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* In some instances, the labeling reaction was halved due to limitations on the amount of DNA available.

Amersham), and 2U of Klenow DNA polymerase (Boehringer Mannheim) were combined and incubated at room temperature for 15 minutes. This was followed by a cold chase (dATP at a C_f of 0.25 mM) for 5 minutes at room temperature. The reaction was quenched by the addition of 40 µl of TE and 10 µl of phenol red. Then, the reaction was loaded onto a Bio-Spin® disposable chromatography column (Bio-Rad) containing packed Biogel P-6 gel (The gel was prepared by suspending it in 1X SSC pH 6.1 and 0.02% sodium azide.). The loaded column was spun for 4 minutes at 2600 rpm and the flow-through containing the labeled probe (~80 µl) was collected. A 1/10 volume of 3 M NaoAc pH 5.2 and 2X 95% ethanol were added, and the tube was placed at –80°C overnight. The next day, the tube was centrifuged for 20 minutes at 14 000 rpm in a 4°C microcentrifuge. The pellet was washed with 75% ethanol, dried, and resuspended in 100 µl of TE. To estimate the amount of radioactivity incorporated into the probe, 1 µl was added to a vial containing 5 ml of Ready Safe™ Scintillation Cocktail (Beckman) and read in a scintillation counter. Probes were stored at -20°C.

Each EMSA reaction was loaded onto a 0.5X TBE 5% polyacrylamide gel (29:1 acrylamide:bis-acrylamide). The gel was pre-run for at least one hour at 150V in 0.5X TBE buffer. The 20-µl reactions consisted of binding buffer (20 mM HEPES pH 7.9, 20 mM KCl, 1 mM spermidine, 10 mM DTT, 10% glycerol, 0.1% NP-40, and fresh 50 mM DTT), 1 µg dIdC (Amersham), and 1 or 2 µg of Dignam nuclear extract from chick retinae (ED7 or ED10). A 50X excess of cold oligonucleotide, AP-2 or SP-1 (Stratagene), was included in two reactions for each probe. The reactions were incubated for 10 minutes at room temperature. One or 2 µl of probe (depending on the activity) was added to the reactions, which were incubated for an additional 20 minutes. Reactions were loaded on the pre-run gel and electrophoresed for 2-6 hours at 150V. A lane containing loading buffer (0.5X TBE, 10% glycerol, 0.25% BPB, 0.25% xylene cyanol) was included to monitor the gel's progress. The gels were dried for 50 minutes at 80°C under vacuum in a gel drier and exposed to X-ray film.

2.5.12. *PCR analysis of chromatin*

Two primer sets were designed to amplify sequence from the *R-FABP* promoter [set one: $^{5'}$GCCTTGTCCTACTGAGCT$^{3'}$(sense) and $^{5'}$ACTGTGGTGCATACTCCC$^{3'}$ (antisense); set two: $^{5'}$CGCCGCTGGGATAATGATG$^{3'}$ (sense) and $^{5'}$CGTCGCGCAGAAAGCCTC$^{3'}$ (antisense)] and one set was made to isolate sequence from the *TH* promoter [$^{5'}$GCTGACTTGTTCTGTTCCC$^{3'}$(sense) and $^{5'}$AGGTGGTGGAGTCATCGTC$^{3'}$ (antisense)]. The reaction components were as described in section 2.4.2, except that 3 μl of input chromatin or 3 μl of chromatin recovered from ChIP was used as the template. The incubation conditions were as follows: *R-FABP* set one, 94°C 5 minutes (94°C one minute, 62°C one minute, 72°C one minute) x 30, 72°C 7 minutes; *R-FABP* set two, 94°C 5 minutes (94°C one minute, 56°C one minute, 72°C one minute) x 35, 72°C 7 minutes; *TH* set, 94°C 5 minutes (94°C one minute, 60°C one minute, 72°C one minute) x 30, 72°C 7 minutes.

2.6. Retroviral-Mediated Gene Transfer Into Chick Embryos

2.6.1. *Vector Construction*

Six constructs were prepared using the RCAS and RCAN system of vectors: (i) and (ii) DNA encoding a GFP and AP-2β fusion protein was inserted into RCAS and RCAN, respectively; (iii) and (iv) DNA encoding the entire AP-2β coding region was introduced into RCAS and RCAN, respectively; (v) and (vi) the coding region for GFP alone was inserted into RCAS and RCAN, respectively. All constructs were first inserted in frame in the pSLAX adaptor plasmid in order to generate unique *Cla* I ends to facilitate cloning into the final RCAS and RCAN vectors.

To prepare (i) and (ii), the coding region of GFP was obtained by cutting the pEGFP-C1 vector (Clontech) with *Eco*R I and *Nco* I. The digest was run out on a 5% polyacrylamide gel, and the 748 bp GFP DNA fragment was excised, electroeluted, and purified. The pSLAX adaptor vector was similarly digested with *Eco*R I and *Nco* I, and gel purified. The GFP insert and pSLAX vector were ligated together. This construct was then digested with *Sma* I and *Eco*R I, and the linearized product was gel purified. The AP-2β coding region was isolated from an available plasmid (pcDNA3) constructed by Dwayne Bisgrove, which contained the complete coding region of this gene inserted into the *Eco*R V site. This plasmid was cut with *Xho* I to liberate the 3' end of AP-2, which was then made blunt with Klenow DNA polymerase: dNTPs (C_f of 0.4 mM) and 6U of Klenow (Amersham) were added to the completed digest, and incubated at 30°C for 15 minutes. The reaction was extracted with organic solvents and incubated overnight at –20°C with a 1/10 volume of 5 M NaCl and 2.5 volumes of 95% ethanol. The DNA was spun, washed, dried, resuspended in TE, and digested with *Eco*R I. After digestion, the AP-2β fragment, with an *Eco*R I 5' end and a blunt 3' end, was gel purified, processed, and resuspended in H_2O. The pSLAX/GFP fragment and the AP-2β fragment were ligated together in a standard ligation reaction.

The AP-2β coding region, prepared above containing the *Eco*R I end and the blunt end, was used in generating the precursor construct for (iii) and (iv). The pSLAX plasmid was digested with *Eco*R I and *Sma* I, gel purified, and resuspended in H_2O. This vector and the AP-2β insert were ligated together as usual.

To generate constructs (v) and (vi), pEGFP-C1 was digested with *Nco* I and *Dra* I to include the stop codon at the end of the GFP coding region. The fragment of interest was excised from a 5% polyacrylamide gel, purified, and resuspended in H_2O. This insert was ligated to pSLAX plasmid that had been digested and purified like the others, except that it was doubly digested with *Nco* I and *Sma* I.

pSLAX constructs with inserts were sequenced with the T3 primer and digested with *Cla* I. These *Cla* I fragments were gel purified and ligated to *Cla* I-digested RCAS and RCAN, which had been dephosphorylated by CIAP, and gel purified. DH5α cells were transformed with the ligation reactions in the usual manner. Plasmid DNA was obtained from the bacterial colonies, digested to verify the correct orientation of the inserts, and sequenced with the 5' RCAS sequencing primer (5'-ACGCTTTTGTCTGTGTGCTGC-3') (Morgan and Fekete, 1996).

2.6.2. *Large-scale Purification of Plasmid DNA*

The six RCAS/RCAN constructs underwent large-scale DNA preparation and purification in cesium chloride. For each plasmid, 330 ml of sterile LB was inoculated with bacteria from a 5 ml seeding culture of DH5α cells transformed with the construct and incubated overnight with shaking at 37°C. The following day, the cells were centrifuged at 6000 rpm for 20 minutes at 4°C. The pellets were resuspended in 30 ml of TEG (stock: 25 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 50 mM glucose) and freshly prepared lysozyme (2 mg/ml final concentration) was added. The cells were incubated for 10 minutes at room temperature. 67 ml of freshly made lysis solution (0.2 N NaOH, 1% SDS) was then added to the cells, which were mixed and incubated on ice for 10 minutes. 50 ml of 3 M potassium acetate pH 4.8 was added to the cells and they were incubated on ice for 15 minutes. The lysed cells were centrifuged at 7000 rpm for 20 minutes at 4°C, and the supernatants were transferred to new vessels by pouring through layers of gauze to filter out excess cell debris. The DNA was precipitated for at least one hour at –20°C in 0.6 volumes of cold isopropanol. The tubes were spun at 7000 rpm for 20 minutes at 4°C and the pellets were resuspended in 3 ml of TE. This was followed by a 2000 rpm centrifugation for 10 minutes at 15°C. 3.2 ml of this supernatant was mixed with 6.5 ml of saturated CsCl and 0.3 ml of ethidium bromide (10 mg/ml) and loaded in an ultracentrifuge tube for a 60 000 rpm spin for 20 hours at 20°C. The broad red band of DNA that formed during this spin was removed. The 60 000 rpm spin was repeated with the recovered DNA and fresh saturated CsCl. The resulting band was removed and extracted with an equal volume of n-butanol. The top layer was discarded, and the extraction was repeated until all the EtBr was removed. The DNA was dialysed in TE (4X with 2L of TE for 1 hour each at 4°C). After the final dialysis, a 1/10 volume of 5 M NaCl and 2 volumes of 95% ethanol were added to the DNA. The tube was mixed and put at –20°C overnight. The tube was spun the following day at 8000 rpm for 20 minutes at 4°C; the pellet was washed in 75% ethanol, dried, and resuspended in TE.

2.6.3. *Construct Testing*

To determine if the constructs were capable of expressing the corresponding proteins, cultures of d5 primary retinal cells were established and transfected with the RCAS/RCAN DNA vectors (see 2.1.12). Chick embryo fibroblasts (CEFs) were also transfected with these plasmids. Whole cell extracts were prepared from the harvested cells and run on protein gels for western immunoblotting.

2.6.3. *Injections*

Embryos at Hamburger and Hamilton stages 11 or 18 (Hamburger and Hamilton, 1951) were injected with the RCAS construct encoding a GFP/AP-2β fusion protein.

Fertilized eggs from White Leghorn chickens were obtained from the University of Alberta farm (Edmonton, AB). The eggs were incubated at 37°C in an egg incubator with 50% humidity until ready for injection. The eggs were swabbed with 70% ethanol, and ~1.5 ml of albumin was removed from the pointed end of each egg with an 18 gauge needle and syringe. A window was cut in the top of the shell with sterile scissors to expose the embryo. Howard's Ringer's solution (60 mM NaCl, 0.8 mM $CaCl_2 \bullet 2H_2O$, 2 mM KCl, pH adjusted to 7.2) containing penicillin and streptomycin was dripped into the window to bathe the embryo and ensure the electrodes were sufficiently immersed in liquid. A solution of plasmid DNA (0.8 μg/μl) and fast green dye (0.1%) in a 15:1 ratio of DNA to dye was loaded into a 1.0 mm glass capillary (World Precision Instruments). The capillary was pulled in advance on a Flaming Brown capillary puller (Sutter Instruments). DNA was then injected directly into either the eye (stage 18) or developing optic vesicle (stage 11) with the aid of a PV 820 Pneumatic Pico-Pump (World Precision Instruments). Following injection, the electrodes, spaced 4 mm apart, were positioned on either side of the injection site and 5 pulses of 50 msec duration were delivered with a 950 msec rest between each pulse. 20V and 25V were delivered to the stage 11 and stage 18 embryos, respectively. A BTX® ECM 830 Electro Square Porator™ was used for the electroporations; the electrodes were custom made out of platinum wire and obtained through Dr. Cairine Logan (University of Calgary, Calgary, AB). After electroporation, the windows of the eggs were sealed with tape and returned to the 37°C incubator.

2.6.4. *Visualization of GFP/AP-2 RCAS Expression*

Infected chick embryos were sacrificed two to five days post-injection and visualized for GFP expression. Eyes and/or retinas were dissected and placed in a Petri dish containing a small amount of PBS. For GFP detection, the FITC filter and fluorescent lamp on an upright microscope was used. Specimens were photographed using Metamorph software.

2.6.5. *Tissue Preservation/Sectioning*

Chick eyes showing GFP expression were immediately placed in 4% paraformaldehyde in PBS and fixed overnight at 4°C. The following day, the eyes were put through sucrose gradients (12%, 16%, and 18% sucrose in PBS). The eyes were incubated for two hours at room temperature in each solution. Individual eyes were then carefully positioned in ~1.5 ml of O.C.T. medium in a tube, flash frozen in a dry ice/methanol bath, and stored at –80°C until ready for sectioning.

Laith Dabbagh (Department of Laboratory Medicine and Pathology, Cross Cancer Institute, Edmonton AB) cut the sections and mounted the slides. Several adjacent sections were taken at various sample depths through the eye at millimetre increments; some sections were destined for H&E staining and others for examination of fluorescent signal. The 8 μm thick sections were cut on a cryostat and mounted on Surgipath® Snowcoat X-tra™ slides. Microscope sections that were H&E-stained were photographed on an Axioskop 2 Plus microscope (Zeiss) using the AxioVision software (version 2.05). The sections that were examined for GFP were fixed in 4% paraformaldehyde for 10 minutes. A 3 to 6 μl aliquot of p-phenylenediamine (PPDA, 1 mg/ml in 90% glycerol) was added to the slide, and a cover slip was placed over the specimen. These slides were visualized with a fluorescent lamp and the FITC filter on an upright Zeiss microscope and photographed using MetaMorph software (version 6.1r2).

CHAPTER THREE

Results of the Experiments to Isolate Genes Regulated by AP-2

A variety of methods were employed to search for target genes of the AP-2 transcription factor in the developing chick retina. These methods included differential screening of a chick retina cDNA library, suppression subtractive hybridization (SSH), searches of the literature, and chromatin immunoprecipitation (ChIP). The results of each of these experiments are presented here.

3.1. Differential Screening of a cDNA Library

Two populations of cells played an essential role in this and subsequent experiments. One population consisted of a d5 primary chick retina cell culture that was transiently transfected with a pcDNA3 expression vector containing an AP-2β cDNA insert. The second population consisted of d5 primary retinal cells transiently transfected with the plasmid containing the AP-2β cDNA in the antisense orientation; thus, AP-2β expression was not expected in these cells as endogenous AP-2 activity is first detected in the chick retina around ED7 (Bisgrove and Godbout, 1999). Dwayne Bisgrove generated these two constructs (*ibid*). A western blot of whole cell extracts from the transfected cells is shown in Figure 3.1.1. Each lane was loaded with 50 μg of protein, and the blot was incubated with anti-AP-2 antibody [anti-AP-2α C-18 rabbit polyclonal antibody (Santa Cruz Biotechnology): this antibody is capable of recognizing both the α and β forms of AP-2 (Bisgrove and Godbout, 1999)]. An AP-2 band denoted by the arrowhead is evident in the sense-transfected lane. The slightly smaller band present in all three lanes is a non-specific protein to which the antibody is binding, and it serves as a control for equal loading of the lanes.

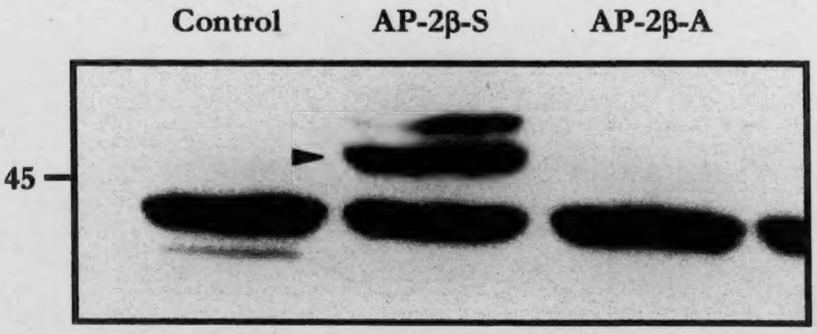

Figure 3.1.1: Western blot of whole cell extracts from the cells used in the searches for AP-2 target genes. AP-2β-S denotes extract from a d5 primary retina cell culture that was transfected with a sense AP-2β expression construct, while AP-2β-A refers to extract from cells transfected with the corresponding AP-2β antisense construct. The control lane contains extract from cells that have been transfected with the empty pcDNA3 expression vector. 50 μg of protein were loaded in each lane, and the blot was incubated with anti-AP-2α C-18 antibody (Santa Cruz Biotechnology). The arrowhead denotes the AP-2 band. The 45 kDa protein standard is indicated on the left.

A flow diagram of the steps involved in searching for target genes of the AP-2 transcription factor is shown in Figure 3.1.2. The Lambda ZAP bacteriophage system (Stratagene) was utilized in the construction of a cDNA library from mRNA isolated from ED7 chick retinae (details of the procedure are described in Godbout, 1992). Plates of infected bacterial cells were prepared, and plaque lifts were performed in duplicate. One filter of a pair was incubated with ^{32}P-labelled cDNA probe synthesized from the d5 primary retinal cultures transfected with the AP-2β sense expression construct, while the other membrane was probed with cDNA obtained from the cultures transfected with the antisense AP-2β construct. Plaques showing differences in signal intensity between the two filters were picked for secondary and tertiary rounds of screening.

The genetic material contained within the selected bacteriophage plaques was then converted to plasmid (pBluescript®) form by *in vivo* excision. The inserts of these clones were sequenced, isolated, labeled with [α-^{32}P]dCTP by nick translation, and used to probe Northern blots. The blots contained poly(A)+ RNA from untransfected ED7 retina, poly(A)+ RNA from the d5 cultures transfected with the sense AP-2β coding sequence, and poly(A)+ RNA from the d5 cells transfected with the vector harboring the antisense AP-2β sequence. Thus, a difference in signal intensity between the last two lanes would be an indication that the expression of the gene being studied was altered by AP-2β. Table 3.1 provides a summary of the clones analyzed in this experiment. In total, eighteen clones recovered from the screen contained insert DNA and were examined by Northern blotting and sequencing. Unfortunately, five clones were very weakly expressed on the Northern blots despite multiple attempts to use them as probes under mild washing conditions; their status with respect to AP-2 regulation could not be determined. Because inserts that showed significant alignments to chicken mitochondrial DNA sequences were considered artifacts,

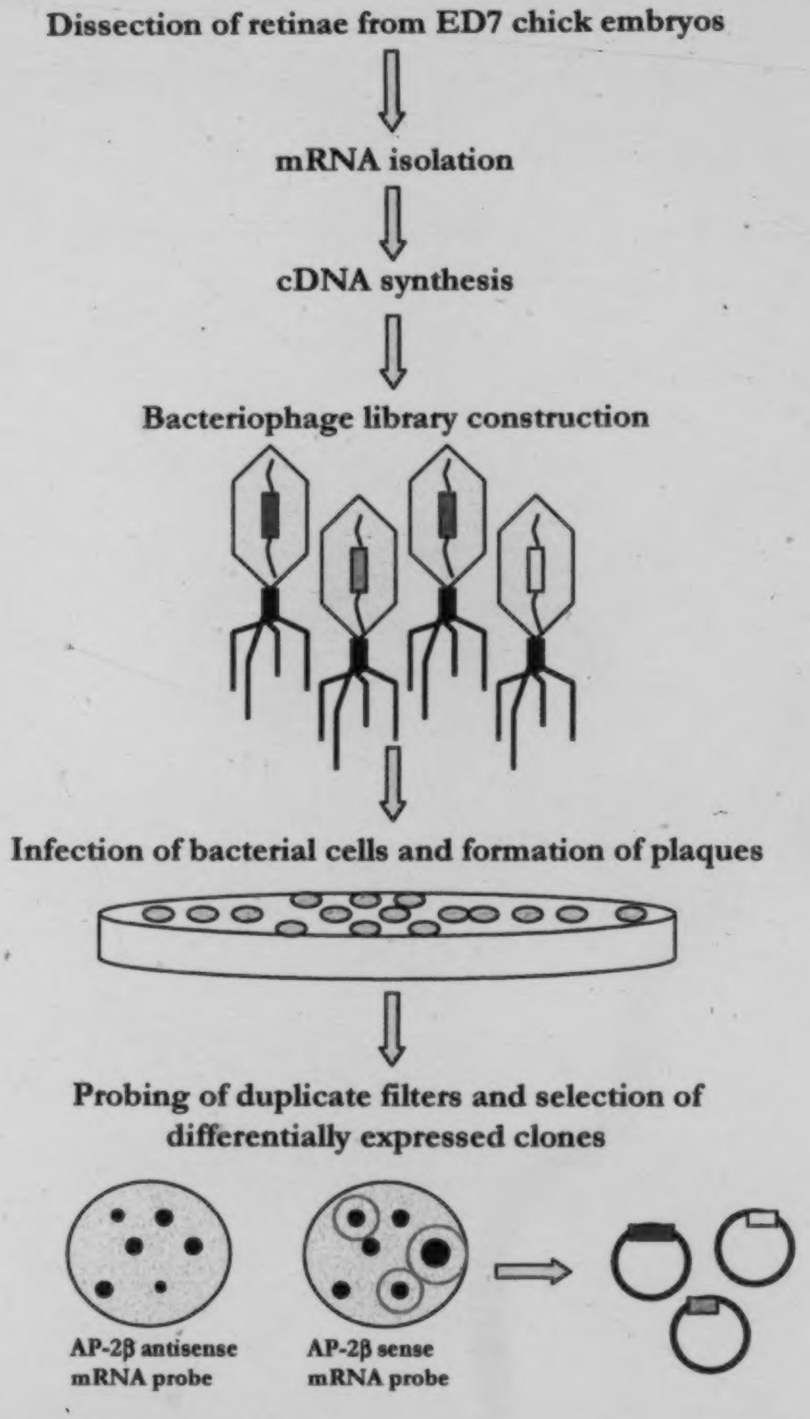
Dissection of retinae from ED7 chick embryos
mRNA isolation
cDNA synthesis
Bacteriophage library construction
Infection of bacterial cells and formation of plaques
Probing of duplicate filters and selection of differentially expressed clones
AP-2β antisense mRNA probe
AP-2β sense mRNA probe

Figure 3.1.2: Flow chart outlining the experiment to find targets of AP-2 by differential screening of an ED7 chick retina cDNA library. See text for details. The isolated clones underwent further analyses.

Table 3.1: Summary of the Analyses of the Clones Identified as Potential AP-2 Target Genes from Differential Screening of a d7 cDNA Library

Clone	Alignments from BLAST searches	Northern Results*
AP-2-1	No significant alignments	Signal weak; appears to be no difference
AP-2-2	*H. sapiens* Zn finger protein 207 (e^{-114})	Slight difference (trial 1) No difference (trial 2)
AP-2-3	No significant alignments	Signal too weak
AP-2-4	*H. sapiens* electron transferring flavoprotein dehydrogenase ($2e^{-11}$)	Signal too weak
AP-2-5	*G. gallus* mRNA for ras-like protein (e^{-157})	No difference
AP-2-7a	Mitochondrial DNA	N/a**
AP-2-7b	No significant alignments	No signal detected
AP-2-9	R. *norvegicus* dnchc2 mRNA for cytoplasmic dynein heavy chain ($9e^{-33}$)	Background too messy
AP-2-10	*H. sapiens* mRNA for KIAA0176 gene, partial cds ($2e^{-23}$)	Slight difference (trial 1) No difference (trial 2)
AP-2-12	Mitochondrial DNA	N/a
AP-2-14	*M. musculus* SMT3B pseudogene ($1e^{-30}$)	No difference
AP-2-16	No significant alignments	Very slight difference
AP-2-18(20)	No significant alignments	No difference
AP-2-20(18)	*G. gallus* T-cell receptor alpha chain gene	Signal too weak
AP-2-22***	*H. sapiens* chromatin specific transcription factor mRNA ($3e^{-20}$)	Slight difference

Table 3.1 (cont.): Summary of the Analyses of the Clones Identified as Potential AP-2 Target Genes from Differential Screening of a d7 cDNA Library

Clone	Alignments from BLAST searches	Northern Results
AP-2-23	*X. borealis* 28S ribosomal RNA gene (e^{-119})	Very slight difference
AP-2-25(27)	Bovine 80-87 kd myristoylated alanine-rich C kinase substrate protein gene	Signal weak, no apparent difference
AP-2-27(25)	Mitochondrial DNA	n/a

NB: Clones in bold denote those for which Northern blot results are shown in Figure 3.1.2.

[*] "Difference" refers to a difference in signal intensity between lanes AP-2β-A and AP-2β-S on the northern blots.

[**] Inserts aligning to mitochondrial DNA in BLAST searches were excluded from the northern analyses.

[***] AP-2-22, when sequenced, showed an alignment to chromatin specific transcription factor mRNA. However, this turned out to be a clone with multiple inserts. The insert that was used on the Northern blot was subcloned and sequenced. This insert turned out to show an alignment to *E. coli* DNA.

and it was predicted that they would be highly expressed on the Northerns, they were excluded from the analysis.

A small number of clones initially seemed interesting, but further investigation eliminated them as good candidates for AP-2 regulation. Examples of Northern blot results from some of these clones are shown in Figure 3.1.3. For instance, insert DNA from clone AP-2-5, which had significant homology to a chicken ras-like protein, shows no difference in signal intensity between the mRNA from AP-2β sense-transfected and antisense-transfected cells. In contrast, probes from clones AP-2-23(b), AP-2-2(c), and AP-2-10(d) all initially showed slight differences between these two lanes; however, no variations in signal intensity were observed upon a subsequent round of hybridization of additional, identical blots with the same probes (data not shown). Because these clones in the end showed no differences in expression following probing of the Northern blots, hybridization with a control DNA sequence (*e.g.* actin) to establish equal loading of mRNA in the lanes, was omitted.

3.2. Suppression Subtractive Hybridization (SSH)

Suppression Subtractive Hybridization (SSH) is a PCR-based subtraction technique that was used to search for AP-2 target genes. A diagram of this procedure is shown in Figure 3.2.1. SSH involves hybridization of the cDNA from two populations of cells, one of which has been affected by a certain variable that induces the expression of a subset of genes. For example, a metastatic cell line can be compared to its non-metastatic counterpart, or cells exposed to hypoxic conditions can be compared to control cells that have been maintained under normal oxygen levels. A subsequent PCR step allows isolation of these genes of interest that are unique to the population being studied. The advantage of this technique over differential screening of a cDNA library is that it should allow the detection of AP-2 target genes of lower abundance.

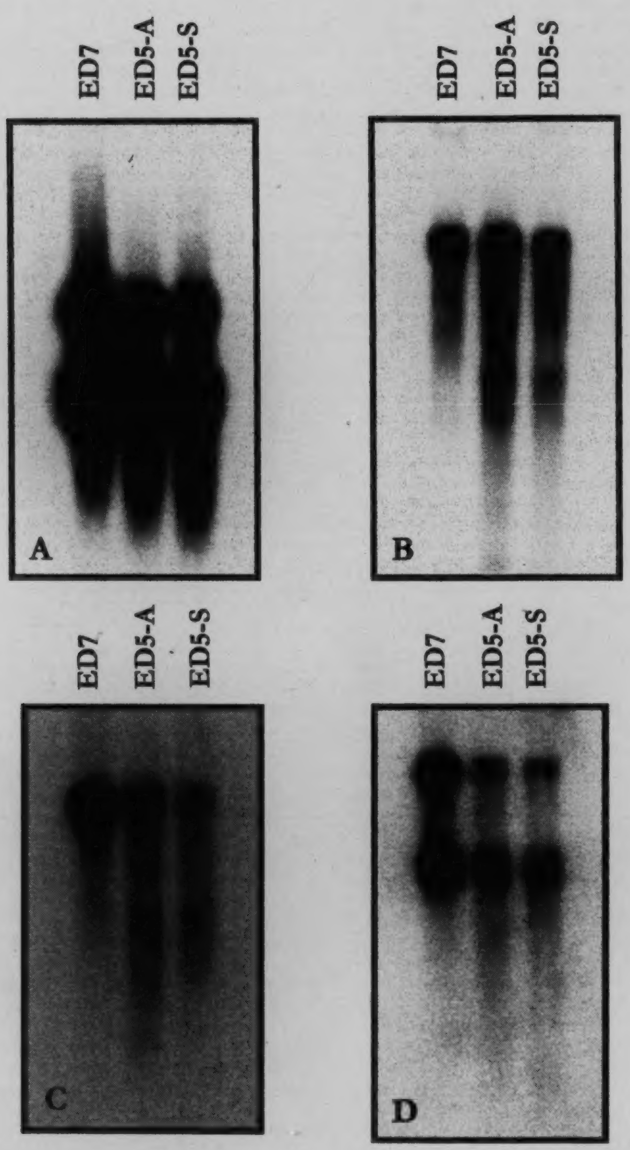

Figure 3.1.3(a-d): Autoradiographs of Northern blots probed with insert DNA from clones isolated by differential screening of an ED7 cDNA library. The lane marked ED7 was loaded with 2 μg of poly (A)+ RNA from ED7 chick retina tissue. ED5-A and ED5-S refer to poly (A)+ RNA (2 μg) from d5 primary retina culture cells transfected with an AP-2β antisense expression construct and a sense expression construct, respectively. **A:** Results of the AP-2-5 probe (showing no difference between lanes ED5-A and ED5-S). **B:** AP-2-23 shows a reduction in signal intensity in the sense mRNA lane versus the antisense mRNA lane. **C:** AP-2-2 shows a difference in signal strength between lanes ED5-A and ED5-S. **D:** AP-2-10 displays a slight difference in the signal intensity of the top band.

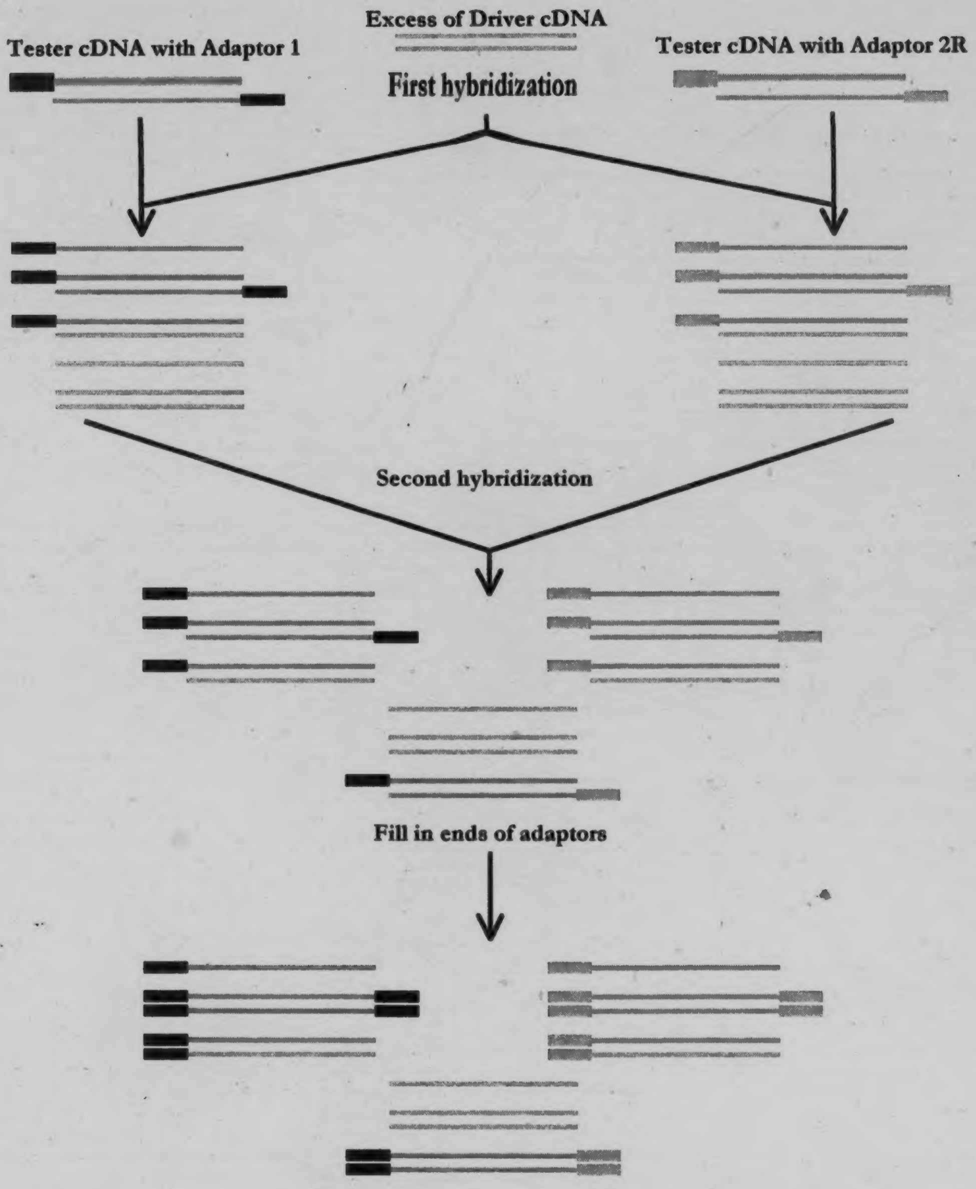

Figure 3.2.1(a): Overview of Suppression Subtractive Hybridization (SSH). In two parallel reactions, tester cDNA (red, obtained from d5 primary retina cultures transfected with a sense AP-2β expression construct) was denatured and hybridized to denatured driver cDNA (green, obtained from similar cultures transfected with the antisense construct). Half the tester had been ligated to Adaptor 1, while the other portion was ligated to Adaptor 2R. A second hybridization was then set up, without denaturing the complexes, in the presence of fresh driver cDNA. The ends of the adaptors were then filled in with *Taq* DNA polymerase, providing a primer binding site for PCR (Figure adapted from Diatchenko *et al.*, 1996).

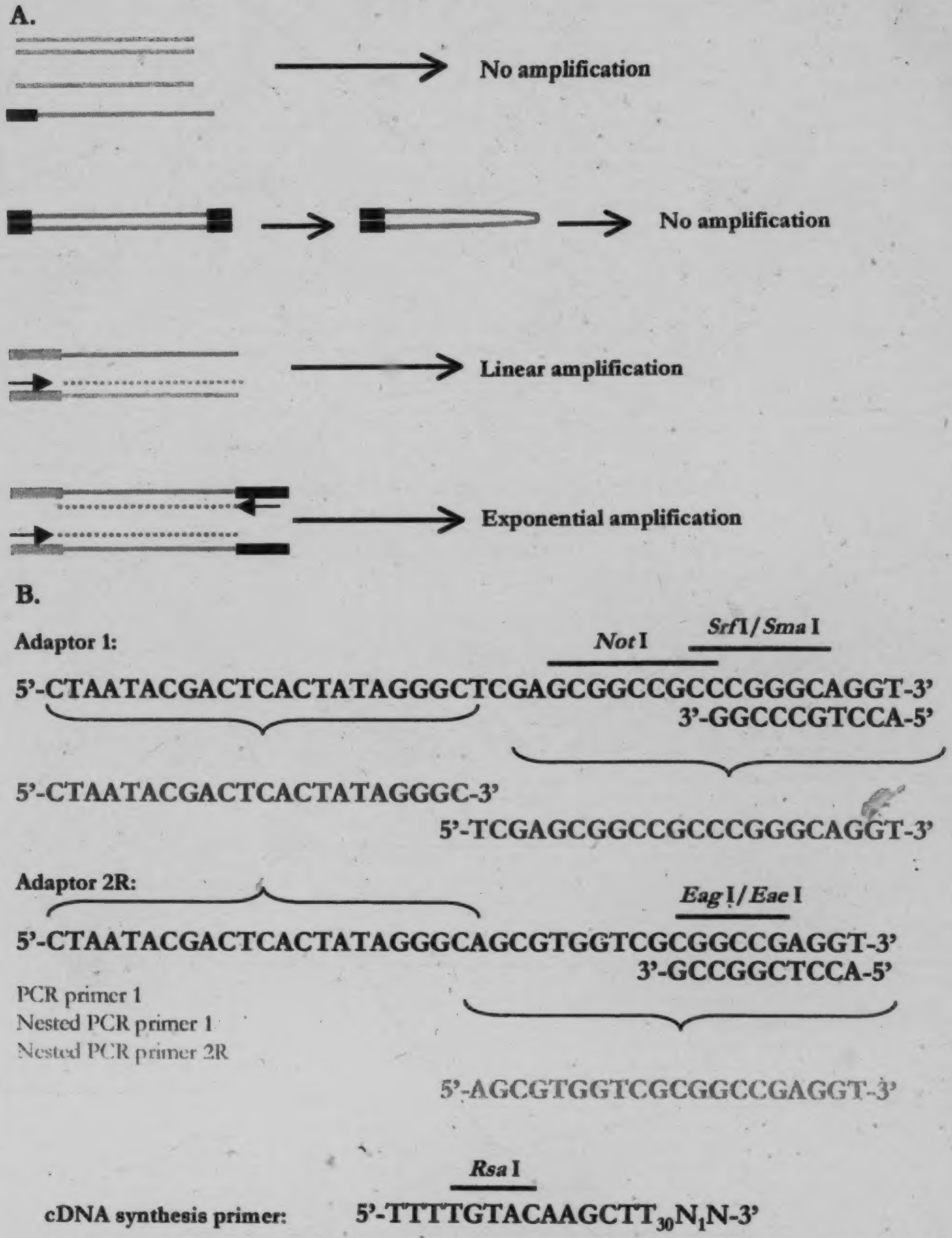
A.
No amplification
No amplification
Linear amplification
Exponential amplification
B.
Adaptor 1:
Not I
Srf I / *Sma* I
5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'
3'-GGCCCGTCCA-5'
5'-CTAATACGACTCACTATAGGGC-3'
5'-TCGAGCGGCCGCCCGGGCAGGT-3'
Adaptor 2R:
Eag I / *Eae* I
5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3'
3'-GCCGGCTCCA-5'
PCR primer 1
Nested PCR primer 1
Nested PCR primer 2R
5'-AGCGTGGTCGCGGCCGAGGT-3'
Rsa I
cDNA synthesis primer:
5'-TTTTGTACAAGCTT$_{30}$N$_1$N-3'

Figure 3.2.1(b): A: The cDNA species that remain following the second hybridization and filling-in of adaptor ends. Only molecules unique to the tester population containing both adaptors (and thus both PCR primer binding sites) are amplified exponentially. Molecules lacking primer binding sites are not amplified, and molecules representing sequences expressed in both populations (with only one adaptor) are amplified at a linear rate. Highly abundant tester sequences are not amplified due to a suppression effect: the homologous ends anneal resulting in a structure that cannot be amplified. **B:** Sequences of the adaptors and primers used in SSH. The restriction enzyme sites are marked (figure adapted from Diatchenko *et al.*, 1996).

Before proceeding with the experiment, the recommended control for the SSH procedure was performed according to directions by Clontech. In brief, cDNA was synthesized from human skeletal muscle poly(A)+ RNA and digested with *Rsa* I. Control DNA, a *Hae* III digest of ϕX174 DNA, was diluted and combined with the skeletal muscle cDNA to become the tester population, which was ligated to the adaptors. An aliquot was removed from each ligation and mixed together to create the unsubtracted tester control. The *Hae* III-digested fragments comprised about 0.2% of the total tester cDNA. The Adaptor 1-ligated tester and the Adaptor 2R-ligated tester were each hybridized to the driver cDNA (human skeletal muscle cDNA that was not ligated to adaptors) and then these reactions were combined. PCR was performed on the unsubtracted tester control and on the subtracted DNA. The products were run out on an agarose gel, and the results are shown in Figure 3.2.2(a). A smear of DNA fragments was observed in the unsubtracted tester control lane, indicating that the ligation worked. As well, a ladder of fragments between 0.2 kb and 1.3 kb, corresponding to the *Hae* III digested ϕX174 bands (found only in the tester cDNA population), is evident in the subtracted control cDNA lane.

Once it was ascertained that the SSH system was working, SSH was performed on the two populations of cells as described in section 3.1. In this particular case, the tester population, expected to contain sequences from genes influenced by expression of AP-2, consisted of cDNA from the d5 population transfected with the AP-2β sense expression construct. Half of this tester cDNA was ligated to adaptor 1 and the other half was ligated to adaptor 2R. The driver cDNA, containing fragments common to both populations, was comprised of mRNA from the cells transfected with the AP-2β antisense construct. The tester cDNA was mixed with the driver cDNA and PCR was performed with primers designed to hybridize to sequences contained in the adaptors (Figure 3.2.1(b)). Primary and

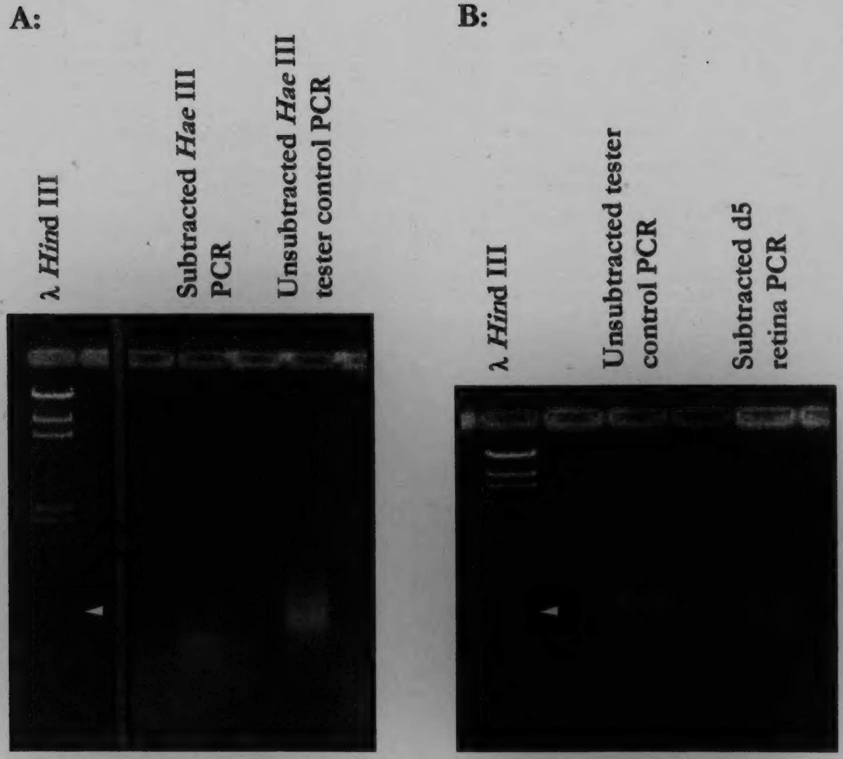

Figure 3.2.2: Agarose gels showing the PCR products from the SSH reactions. **A:** Results of the control SSH reaction. Bands corresponding to the *Hae* III digested ϕX174 DNA bands are evident in the lane marked subtracted *Hae* III PCR. A smear of DNA fragments in the unsubtracted *Hae* III tester control lane indicates the ligation of adaptors to the cDNA was successful. **B:** Results of the SSH experiment with d5 chick retina cDNA. Again, a smear of fragments in the unsubtracted tester control PCR lane shows that the adaptor ligation was successful. Products are also observed in an aliquot of the PCR reaction following SSH in the subtracted d5 retina PCR lane. These fragments were cloned into pBluescript® for further analysis. In both **A** and **B,** the λ *Hind* III ladder (fragments from top to bottom: 27 500 bp, 9400 bp, 6500 bp, 4300 bp, 2300 bp, 2000 bp, and 560 bp) has been included in the lane on the left. The white arrowheads denote the faint 560 bp bands.

secondary amplification reactions were both performed with primer 1, as one of the nested primers had an unusually high Tm: reactions using the nested primer set proved unsuccessful. As well, PCR analysis was done on the unsubtracted tester control (tester cDNA with no driver cDNA added) to test the efficiency of the adaptor ligation. Figure 3.2.2(b) shows the results of the PCR reactions. Again, the DNA in the unsubtracted tester control lane provides evidence that the adaptor ligations were working, while the smear in the d5 subtracted retina lane showed that PCR fragments were retrieved following the SSH procedure. These resulting fragments were cloned into pBluescript®, and 105 clones were picked from this library for plasmid DNA preparation.

Insert DNA from these SSH clones was loaded in equal amounts onto duplicate slot blots for hybridization with labeled cDNA probes, from the two transfected cultures, in order to confirm that the clones obtained through SSH were, in fact, differentially expressed between the two mRNA populations compared in the experiment. Slot blotting was the method of choice for screening the SSH clones, as many DNA samples could be analyzed on a single blot. As well, the results from DNA blotting with a manifold and vacuum tend to be more reliable than a method such as dot blotting, due to elimination of uneven DNA spreading among samples, which makes quantification of signal more difficult. During initial experiments, it was determined that a method involving SSC transfer of the fragments produced better results than a transfer in alkaline buffer (data not shown). Isolated DNA inserts prepared by electroelution were used on the blots, as the pBluescript® vector at ~3 kb in size was many times greater than the average expected size of the SSH-DNA fragments (*i.e.* *Rsa* I has a recognition sequence 4 bp in length, so it should cut DNA approximately once every 256 bp.). Several DNA controls (*e.g.* plasmid DNA, DNA fragments from genes not expected to be differentially expressed between the two blots) were included to normalize for differences in autoradiography in the event that the probes labeled with different efficiencies. One blot in the pair was probed with radioactively labeled cDNA obtained from d5 primary chick retinal cultures transfected with the vector containing the AP-2β coding region. The other blot was probed with cDNA from the d5 cultures transfected with the corresponding antisense AP-2β construct. Representative blots are shown in Figure 3.2.3. The blots marked A1 and A2 are a pair while B1 and B2 are another. A1 and B1 were probed with cDNA from the antisense-transfected cells, and A2 and B2 were probed with cDNA from the sense-transfected cultures. The bottom row of slots includes the control DNA mentioned above. These lanes include, from left to right, pGAPDH sequence, actin sequence, and pBluescript® vector DNA. The red arrowheads indicate clones that showed differences in signal intensity between the blots by visual inspection.

The inserts that showed a difference in signal strength between the two blots were selected for further analysis. In total, less than 20% of the clones examined displayed such differences. These clones were sequenced, and the insert DNA, labeled with [α-^{32}P]dCTP by nick translation, was used to probe Northern blots as already described in section 3.1. Representative Northern blots are shown in Figure 3.2.4. None of the autoradiographs showed greater than two-fold differences in signal intensity between the sense and antisense-transfected lanes. Slight differences (two-fold or less) were observed for probes 6 (top band), 46 (top band), 102, 52, and 53. No differences were noted for probe 104. Re-probing the blots with an actin probe revealed little lane variation. Table 3.2 contains a summary of all the analyzed SSH clones. A number of clones showed either no difference between the two transfected mRNA lanes on the blot or no apparent signal on the blot. A few clones showed differences between the two lanes but some of these were judged

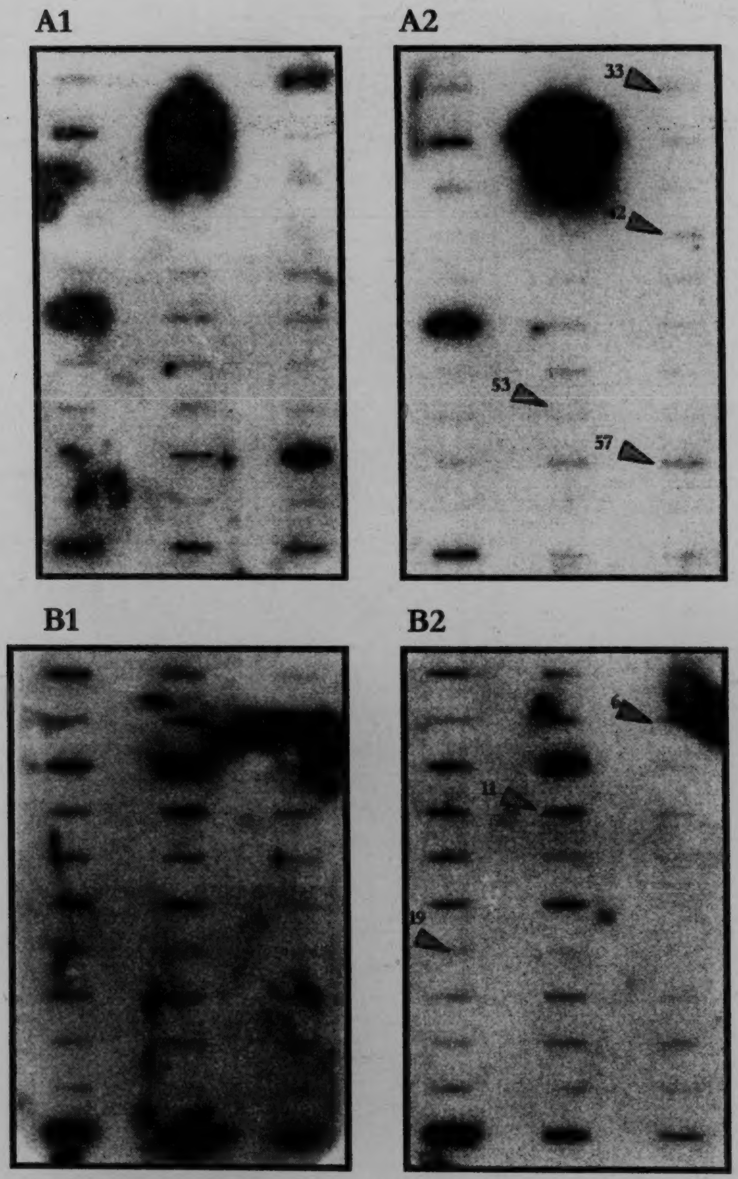

Figure 3.2.3: Pairs of representative slot blots. Identical slot blots were constructed with insert DNA from clones recovered from the SSH experiment and probed in order to identify differentially expressed clones. Blots A1 and A2 are replicates as are blots B1 and B2. A1 and B1 were probed with radioactively labeled cDNA from d5 primary retinal cell cultures that have been transfected with the AP-2β antisense expression construct. A2 and B2 were probed with labeled cDNA from cultures transfected with the sense construct. Clones that showed a difference in intensity between the blots (examples are denoted by the red arrowheads) were selected for further analysis. The bottom row in each blot contains pGAPDH DNA, actin DNA, and pBluescript® vector DNA used as controls for differences in intensity of labelling between paired blots.

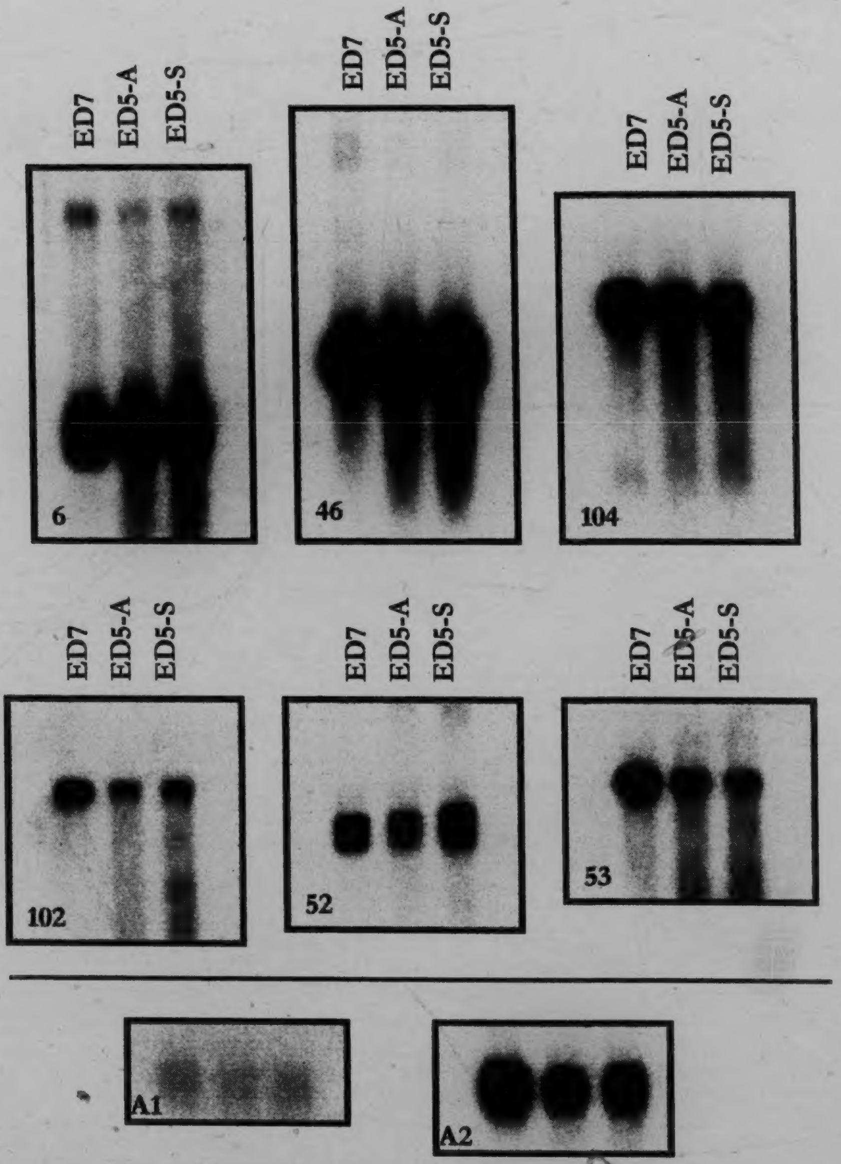

Figure 3.2.4: Example autoradiographs from Northerns probed with insert DNA from the clones isolated by SSH. The lane marked ED7 was loaded with 2 μg of poly (A)+ RNA from ED7 chick retina tissue. ED5-A and ED5-S refer to mRNA (2 μg/lane) from d5 primary retina culture cells transfected with an AP-2β antisense expression construct and a sense expression construct, respectively. Most of the differences observed between the two transfected lanes are very slight. A1 and A2 represent actin controls of two of the blots. A1 is the blot that corresponds to 53 and 104 while A2 is the blot that was used for clones 6, 102, and 46.

Table 3.2: Summary of the SSH Clones Selected for Further Analysis Following Slot Blotting

Clone	Alignments from BLAST Searches	Northern Results*
6	cDNA clone from chicken fat library similar to S19 ribosomal protein ($4e^{-19}$)	Very slight difference
11	Unidentified *Gallus gallus* clone from liver library (e^{-137})	No difference
19	Various *M. musculus* cDNA clones ($4e^{-36}$)	Very weak signal
33	*G. gallus* cDNA clone 30f20r1 from bursa of Fabricius library (e^{-117})	Very weak signal; no apparent difference
38	Mitochondrial genome	Slight difference
42	*G. gallus* cDNA clone ROS056H05 (e^{-110}) from Stratagene Chick Embryo Lambda cDNA Library	No difference
46	Chicken mitochondrial genome	Very slight difference
49	No significant alignments	Very slight difference
50	*G. gallus* clone from liver library similar to MHC B12 alpha-chain (e^{-40})	Very slight difference
52	No significant alignments	Slight difference
53	Chicken liver cDNA library clone similar to GNEF p532 ($2e^{-23}$)	Very slight difference; not reproducible on later trials
57	*G. gallus* clone from liver library similar to H. sapiens ribosomal protein S23 (e^{-113})	Slight difference
62	No significant alignments	Very weak signal; no apparent difference
75	Sequence data poor—no significant alignments	Signal too weak

Table 3.2 (cont.): Summary of the SSH Clones Selected for Further Analysis Following Slot Blotting

Clone	Alignments from BLAST Searches	Northern Results*
92	No significant alignments	Signal too weak
94	Similar to chicken mitochondrial DNA clones	No difference
102	*H. sapiens* IGSF4 gene ($6e^{-38}$)	Very slight difference
104	Data not available	No difference

NB: The results for the clones in bold typeface are shown in figure 3.2.2.

*"Difference" refers to a difference observed in signal intensity between the lane containing mRNA from d5 primary retinal cell cultures transfected with a sense AP-2β expression construct and the lane containing mRNA from the cells transfected with the antisense AP-2β construct.

unlikely to be good candidates based on their identities. Clone 46, in particular, represented a mitochondrial DNA gene.

3.3. RT-PCR of Genes Identified as AP-2 Targets From the Literature

The chicken choline acetyltransferase (*ChAT*, GI: 15341175), dopamine β-hydroxylase (*DBH*, GI: 6983691), and tyrosine hydroxylase (*TH*, GI: 6523292) genes were identified from the literature as potential candidates for AP-2 regulation. The criteria used for identifying candidates were that (i) the genes contained at least one identified AP-2 binding site in their promoter region, and (ii) the genes were known to be expressed in the retina, particularly in horizontal and amacrine cells (see Kim *et al.*, 2001; Baskin *et al.*, 1997; Dyer and Cepko, 2001; and Chen *et al.*, 1999). RT-PCR was performed using first strand cDNA from ED10 or ED16 chick retinae and primer sets that were designed to isolate sequence from the coding region of each of these genes. The choice between using ED10 or ED16 retinae in the reaction was empirically determined. The resulting PCR products were run out on agarose gels to confirm their sizes and cloned into the pCR®II-TOPO® vector. The plasmids were then sequenced to verify the identities of the fragments. The three PCR inserts liberated from the pCR®II-TOPO® plasmid by digestion with *Eco*R I are shown in Figure 3.3.1. As expected, the *ChAT*, *DBH*, and *TH* fragments are ~520 bp, 460 bp, and 900 bp in size, respectively.

3.3.1. *Northern blot analyses*

DNA from each cloned PCR product was obtained through electroelution, and radioactively labeled by nick translation in preparation for hybridization to a Northern blot (as in section 3.1) to determine if these genes were AP-2 regulated. Unfortunately, choline acetyltransferase and tyrosine hydroxylase did not display any detectable signal, despite being

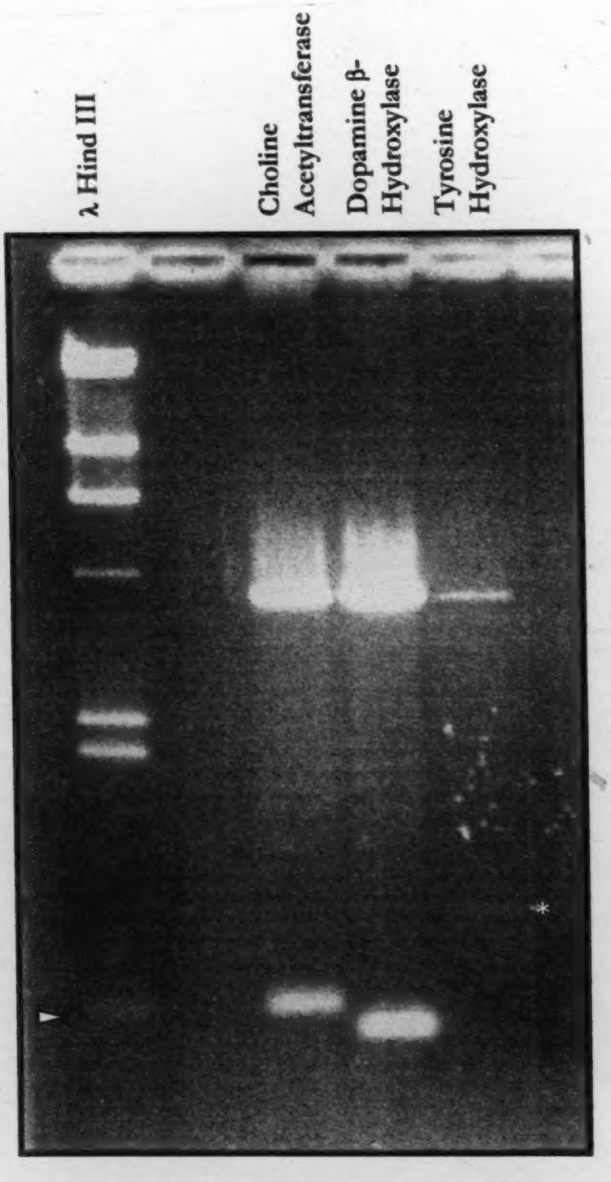

Figure 3.3.1: Agarose gel showing the results of a restriction endonuclease digestion of the three RT-PCR products cloned into the pCR®II-TOPO® vector. The three digests are from left to right, choline acetyltransferase, dopamine β-hydroxylase, and tyrosine hydrosylase with inserts of ~520 bp, ~460 bp, and ~900 bp, respectively. The λ *Hin*dIII ladder is shown on the far left. The 560 bp fragment is denoted by the white arrowhead. The tyrosine hydroxylase preparation in this case was very dilute and it was later repeated (data not shown). The asterisk denotes the weak insert band obtained for tyrosine hydroxylase. The DNA insert in the lane corresponding to choline acetyltransferase appears to run more slowly than the 560 bp ladder fragment due to loading this lane with an excess of DNA.

washed at a lower temperature and exposing the blot to film for several days at –80°C (data not shown). Thus, the regulation status of these genes with respect to AP-2 could not be determined. In contrast, a faint signal was evident on the blot when it was probed with the dopamine β-hydroxylase DNA; however, while the signal appears stronger in cells transfected with the AP-2β sense construct, it is still too faint to make any definitive conclusions. The result from this autoradiograph is shown in Figure 3.3.2.

3.4. Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was another technique used to search for and clone novel AP-2 target genes in the developing chick retina. ChIP is performed *in vivo*, rather than *in vitro* like the other methods described thus far. ED10 chick retinae were used for ChIP as AP-2 expression in the retina peaks around this stage (Bisgrove and Godbout, 1999). It was reasoned that using tissue at this stage would therefore increase the probability of finding AP-2 targets. Figure 3.4.1 briefly outlines the ChIP technique. In essence, cells of interest are treated with formaldehyde to cross-link the proteins to DNA in the cell. The cells are lysed, and the DNA is sheared by sonication into fragments of manageable sizes. An immunoprecipitation is then performed, by adding an antibody that binds to the protein of interest, and the complex is retrieved with protein A or G sepharose beads. The recovered protein:DNA complexes are then treated to undo the cross-links, and the DNA is isolated for further manipulations, namely PCR or cloning.

3.4.1. *Chromatin Immunoprecipitation (ChIP) Results*

Before ChIP reactions were performed with the ultimate goal of cloning the resulting AP-2 target gene fragments, numerous reaction conditions needed to be sorted out. For instance, critical components to be established were the length of the incubation and the

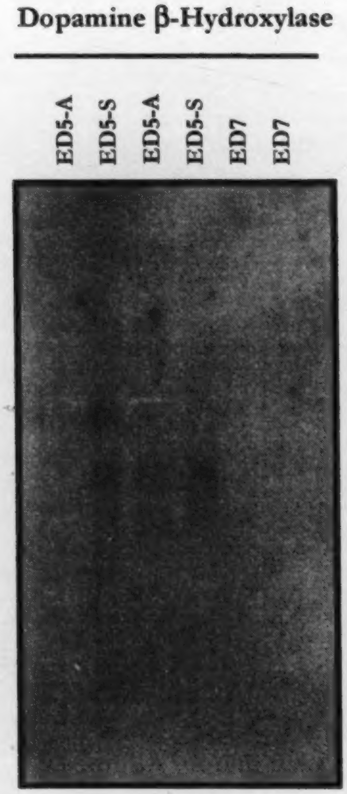

Figure 3.3.2: Autoradiograph of a Northern blot probed with the RT-PCR product from dopamine β-hydroxylase. Faint signal is evident with this probe. ED7: mRNA from ED7 chick retina cells; ED5-A: mRNA from d5 primary retinal cell cultures transfected with an expression plasmid containing the antisense AP-2β sequence; ED5-S: mRNA from d5 primary retinal cultures transfected with the expression plasmid containing the coding region for AP-2β (sense orientation).

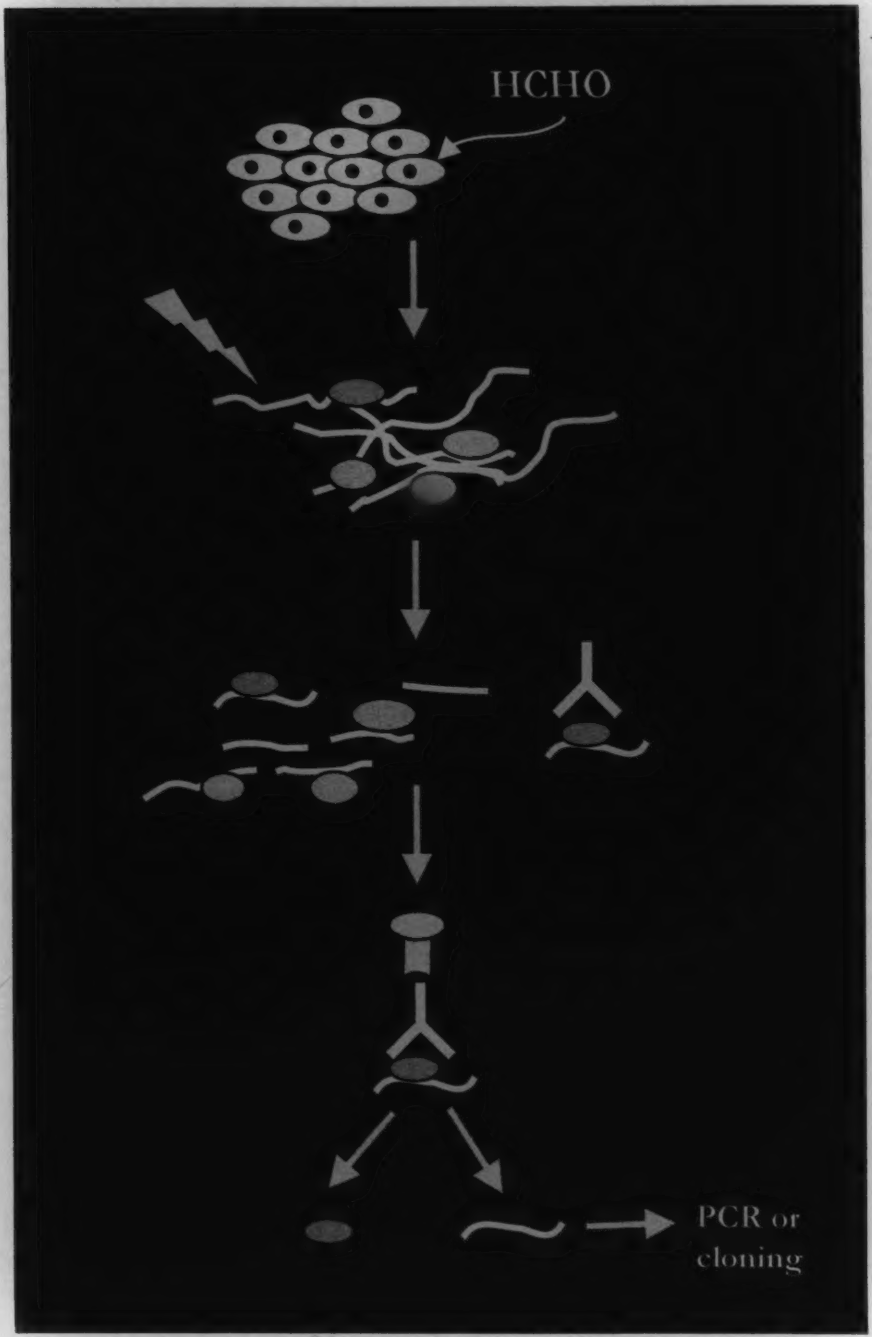

Figure 3.4.1: Summary of the chromatin immunoprecipitation reaction (ChIP). Cells are fixed in formaldehyde to cross-link proteins to DNA. The cells are lysed and sonicated to shear the DNA. An IP is performed with an antibody of interest and protein A sepharose beads. The DNA:protein complexes are eluted, the cross-links are reversed, and the DNA is recovered for further analyses, either PCR or cloning.

concentration of formaldehyde used for the cross-linking step. Insufficient cross-linking would result in no DNA being recovered with the protein in the immunoprecipitation, while excessive cross-linking would result in complications such as a significant reduction in the amount of DNA shearing during sonication. Figure 3.4.2(a) shows the results of one of our preliminary experiments. Under the same sonication conditions, DNA fragments from retinal cells treated with HCHO for 5 minutes were significantly smaller than those observed from cells treated for 15 minutes. In fact, in the 15-minute treatment, no visible differences in shearing were observed between a 30-second pulse and five 30-second pulses. We then implemented a more methodical approach to arrive at optimal cross-linking conditions for ChIP. Various combinations of HCHO concentration and incubation times were examined: no formaldehyde, a 2 minute treatment with 1% HCHO, 5 minutes with 1% HCHO, 15 minutes with 1% HCHO, 10 minutes with 0.1% HCHO, and 10 minutes with 0.5% HCHO. The reactions were layered on top of a cesium chloride gradient for a 20-hour spin in an ultracentrifuge. After centrifugation, free DNA is expected to end up near the bottom of the tube, DNA:protein complexes near the middle, and free protein on top. Fractions of ~400 μl were collected through a hole in the bottom of the tube, and an aliquot from each fraction was run on a 1% agarose gel. Protein:DNA complexes were represented by a bright band near the well, and free DNA consisted of a smear throughout the lane. It was determined that a 5 minute treatment with 1% formaldehyde was sufficient to bind most of the DNA to the proteins [Figure 3.4.2(b)]. Yet, these complexes were not so big so as to be unmanageable during sonication.

Suitable conditions for the DNA sonication step were then empirically determined before proceeding. ChIP reactions were set up with various conditions for sonication (*i.e.* the duration, output setting, and number of pulses were varied). A long sonication followed

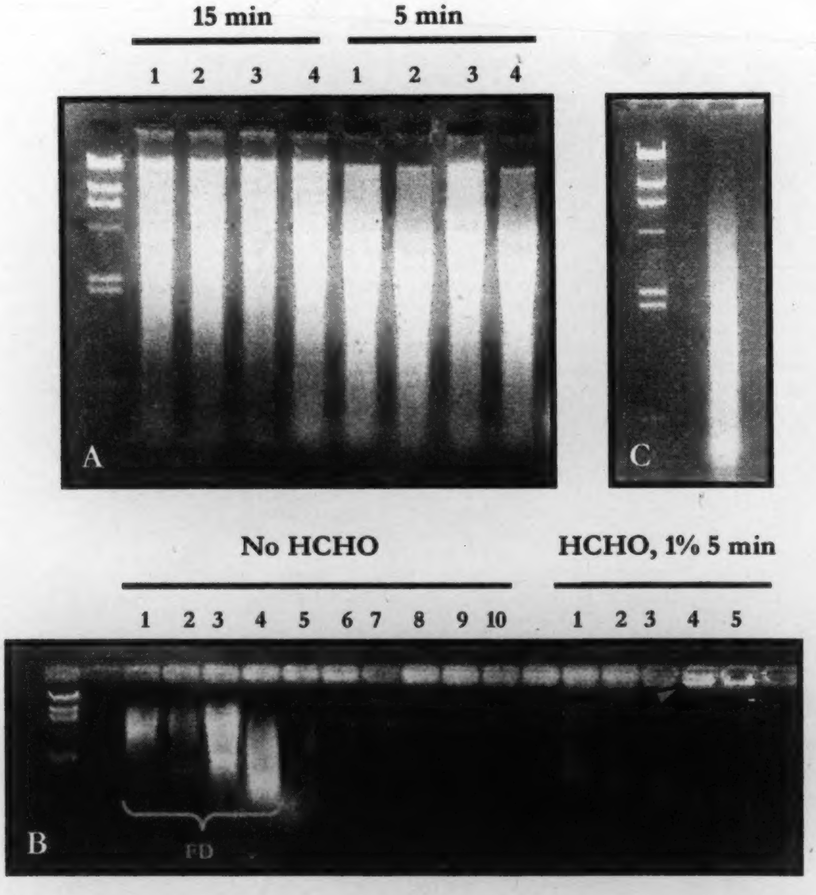

Figure 3.4.2(a-c): Results of experiments to optimize cross-linking and sonication conditions. **A:** Agarose gel of sonicated DNA fragments. Retinal cells were treated with 1% formaldehyde for either 15 minutes or 5 minutes and sonicated for various durations on setting 5 at 50% duty of a Branson Sonifier® Cell Disruptor 200 (1: 2X 10 seconds; 2: 6X 10 seconds; 3: 1X 30 seconds; 4: 5X 30 seconds). **B:** Gel of the fractions collected after cross-linked and non-cross-linked samples of nuclear extracts were spun on a CsCl gradient. The number of the lane refers to the number of the fraction with "1" beginning at the bottom of the tube. The lanes corresponding to fractions 6-10 of the 1% 5-minute HCHO trial were empty and they are not shown. A DNA:protein complex in a fraction from the middle of the gradient is denoted by the red arrow while free DNA (FD) is denoted by the bracket. **C:** Gel showing the distribution of DNA fragments under the sonication conditions used for the ChIP experiments.

by two shorter ones (*e.g.* 30 seconds, then 2X 15 seconds) seemed to be most efficient [The length of time of the first burst seems to have the greatest effect on sonication (B. Bobechko, University of Toronto, Toronto ON, personal communication).]. The IP step was omitted, and the procedure was resumed at the cross-link reversal step. The final chromatin samples were resuspended in H_2O, the amount of DNA in the samples was estimated by spectrophotometry, and several micrograms of DNA were run on an agarose gel to examine the size distribution of the DNA fragments. In this manner, it was determined that on a Sonifier® Cell Disruptor 200 (Branson) with a 2 mm microtip, a 30 second burst followed by two 15 second bursts at 50 percent duty in continuous mode on setting 6 achieved close to the desired distribution of fragments [Figure 3.4.2(c)].

The efficacy of the antibody to be used also required testing before proceeding with ChIP. Immunoprecipitations were performed on Dignam ED10 chick retina nuclear extracts and on nuclear extracts from retinal cells that had been cross-linked in a ChIP experiment. In the former case, 1 ml of ChIP dilution buffer was added to ~200 μg of nuclear extract. Figure 3.4.3(a) shows that anti-AP-2 antibody (anti-AP-2α rabbit polyclonal antibody C-18 lot A247, Santa Cruz Biotechnology) is capable of retrieving AP-2 protein from ED10 nuclear extract, as a band of approximately 52 kDa, the size of AP-2, is present in the IP lane. This band is also observed in the lane containing total nuclear extract, loaded as a positive control for immunoblotting. Figure 3.4.3(b) shows that upon introducing the cross-linking component to the IP reaction, AP-2 protein is still recovered with this antibody as evidenced by a band of ~52 kDa following immunoblotting. The results of IPs performed with pre-immune rabbit anti-serum as a negative control are also shown in Figure 3.4.3(a, b): no AP-2 was recovered with pre-immune serum. Figure 3.4.3(c) shows a blot of the IP results using a second AP-2 antibody in a ChIP reaction (anti-AP-2α H-79 rabbit

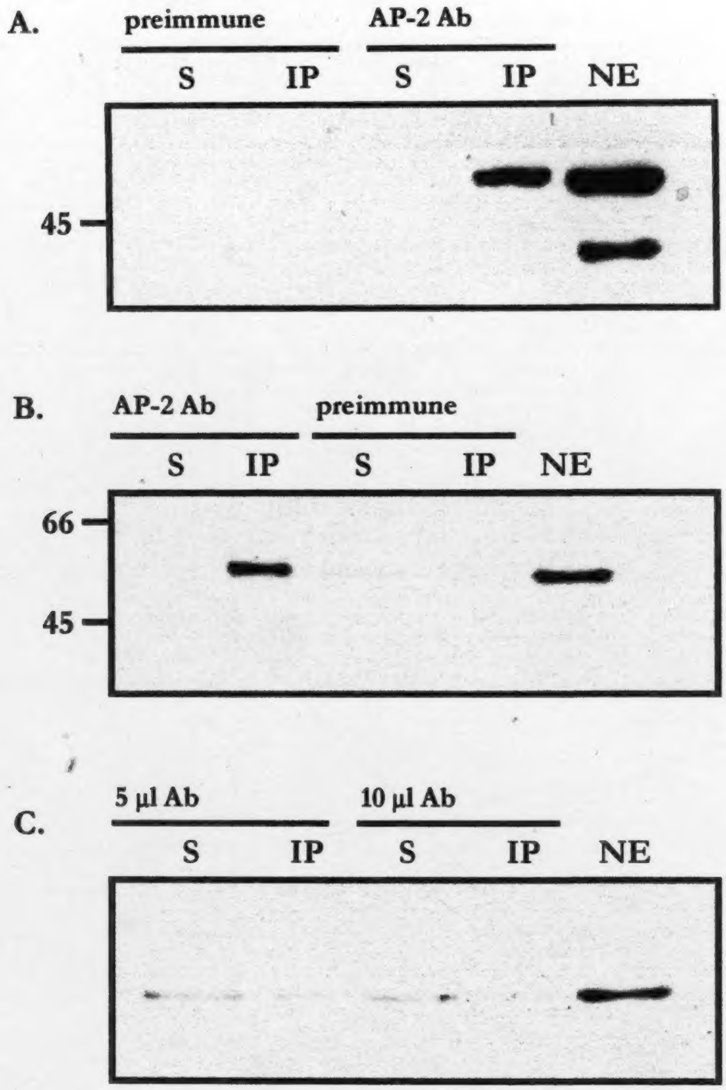

Figure 3.4.3(a-c): Western blots of AP-2 immunoprecipitations. **A:** Immunoprecipitation of AP-2 from ED10 chick retina nuclear extract (Dignam) using a rabbit polyclonal anti-AP-2 antibody. **B:** Immunoprecipitation of AP-2 from ED10 chick retina cells that were treated with formaldehyde (1% for 5 minutes) in order to cross-link proteins to DNA. This IP was performed following the ChIP protocol with the same antibody as in A. **C:** Immunoprecipitation of AP-2 from formaldehyde-treated ED10 chick retina cells. The antibody used, in quantities of 5 and 10 μl, was AP-2α H-79 rabbit polyclonal antibody (lot L061 Santa Cruz Biotechnology). Immunoblotting in all instances was carried out with a mouse monoclonal AP-2 antibody. Molecular weight markers in kDa are indicated on the left. A preimmune antiserum control is also shown in blots A and B. Residual AP-2 in supernatants following the IP is shown in the lanes marked S. 40 μl of supernatant was loaded in each of these lanes. Lanes labeled NE contain 50 μg of total nuclear extract (Dignam) from ED10 chick retina.

polyclonal antibody lot L061, Santa Cruz Biotechnology). In this case, the recovered AP-2 band from the IP, while still present, is not as dramatic as in the first two blots. Most of our experiments were carried out with the H-79 antibody as we ran out of the C-18 antibody after carrying out all the preliminary experiments. Testing of different lots of the C-18 antibody yielded poor results. To circumvent the drawbacks of using a less efficient antibody for ChIP, numerous IP reactions were performed and the products were pooled. All blots were incubated with a mouse monoclonal AP-2 antibody [3B5 generated by Dr. Trevor Williams (Yale University, New Haven, CT) obtained through the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA)].

Once the ChIP reaction was performed, *EcoR* I linkers were phosphorylated with tracer amounts of [γ-^{32}P]ATP and ligated to the recovered ChIP DNA in preparation for cloning. The rationale behind using this strategy was two fold. First, linkers with *EcoR* I sites could be cut with this enzyme to produce cohesive ends, thus facilitating cloning, as ligations with blunt ends are much more difficult. Second, the radioactively labeled linkers could be used to track the ChIP DNA, expected to be present in very small quantities, in further manipulations. The autoradiographs of two gels are displayed in Figure 3.4.4. The gel on the left was constructed to test the success of the polynucleotide kinase reaction to label the linkers. Two aliquots of labeled linkers are shown on the gel, which migrate more slowly than a dilution of free [γ-^{32}P]ATP. Lanes showing i) a ladder of linker concatemers following a linker ligation reaction and ii) an *EcoR* I digest of a portion of these concatemers are also included. These two reactions were included as controls to ensure that the linker ligation reaction was working and that these linkers were capable of undergoing digestion. The gel on the right shows two aliquots of ChIP DNA (each comprising ~10% of the total reactions) that have been ligated to the linkers; the one on the left has been digested with

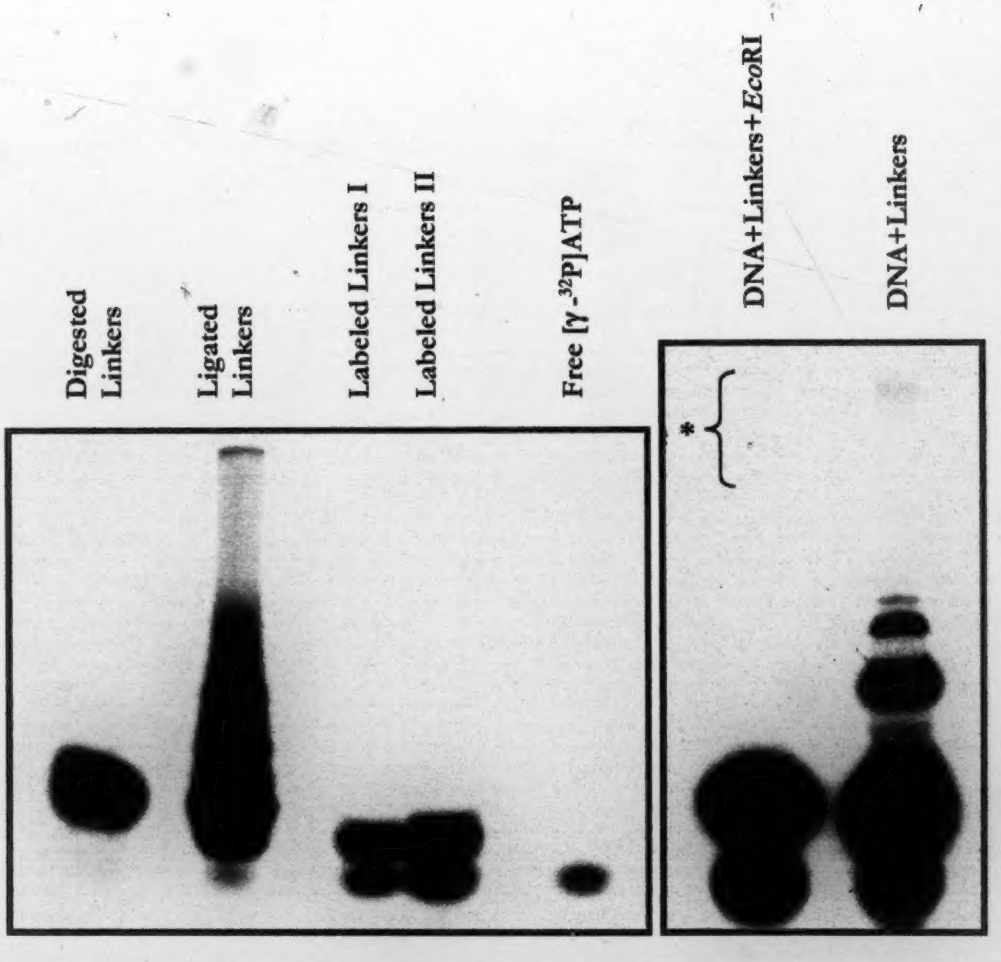

Figure 3.4.4: Left: Autoradiograph showing test results of the polynucleotide kinase reaction to label *Eco*R I linkers with [γ-^{32}P]ATP in preparation for the cloning of ChIP fragments. From *right* to *left*, the lanes include the following: a 1/50 000 dilution of [γ-^{32}P]ATP, two lanes of phosphorylated linkers after the kinase reaction, a portion of a ligation reaction of labeled *Eco*R I linkers (to create concatemers), and several microlitres of a digest of the labeled linker concatemers. **Right:** Autoradiograph showing the results of the ligation of labeled linkers to ChIP DNA fragments. The lane on the right includes 1/10 of the ligation reaction, while the lane on the left was loaded with 1/10 of the subsequent *Eco*R I digestion. The asterisk indicates the approximate size range of the fragments (*i.e.* greater than 250 bp) that were excised from another lane on the gel (not shown) that contained the remainder of the digestion reaction.

*Eco*R I. The fragments designated by the asterisk (*i.e.* greater than ~250 bp in size) were excised from a lane containing the remainder of the digested ChIP DNA with *Eco*R I ends and purified. The small amount of signal in this lane was an encouraging indication that DNA was, in fact, recovered from the ChIP reaction for cloning.

Following purification, the ChIP DNA was cloned into pBluescript®, and plasmid DNA was obtained from selected clones. In total, 59 clones were sequenced. Nearly 80% of these clones did not reveal any similarities to archived material in databases through BLAST searches. The sequences from these clones were then analyzed for putative AP-2 binding sites. The programs used included GeneTool (www.biotools.com), AliBaba2.1 and Patch (TRANSFAC database, www.gene-regulation.com) and Tfsitescan (MIRAGE database, www.ifti.org). 35 clones contained at least one prediction of an AP-2 binding site by these programs. The majority of these clones with predicted binding sites underwent EMSA analyses, discussed below.

Finally, PCR was attempted on a portion of the recovered DNA from ChIP to ascertain if sequence from genes that are known or suspected to be AP-2-regulated could be recovered in the ChIP reaction. Primers were designed to flank the AP-2 binding sites in the promoter regions of the *R-FABP* and tyrosine hydroxylase genes. Unfortunately, sequence from these genes was not recovered following PCR, although they were able to amplify product of the expected size from a preparation of chicken genomic DNA (data not shown). It remains to be seen if adjustments to the reaction conditions or new primer sets may be able to amplify sequence from these promoters following ChIP.

3.4.2. *EMSA Analyses*

To verify that the sites identified by the computer programs were *bona fide* AP-2 binding sites, EMSA was carried out for each clone that had at least one predicted AP-2 binding site within its sequence. In total, 32 EMSA gels were run, and the autoradiographs of these gels are shown in Figure 3.4.5(a-p). Figure 3.4.5(a) contains an example of what one can expect to see on a typical gel. The first lane contains labeled probe to which no protein has been added. The arrow indicates the position that this probe has migrated to in the gel. Lanes two and three contain labeled probe pre-incubated with ED10 nuclear extract (2 μg and 1 μg, respectively). When protein binds to the DNA, a higher molecular weight complex is formed that migrates more slowly through the gel in comparison to the free DNA. The blue arrowheads indicate the position of these protein:DNA complexes. The fourth and fifth lanes contain labeled probe pre-incubated with 1 μg of ED10 nuclear extract and a 50X molar excess of a consensus AP-2 binding site and SP-1 binding site, respectively. The sixth lane contains probe that has been pre-incubated with 2 μg of ED7 nuclear extract, while the last lane contains probe that was pre-incubated with 1 μg of this extract. In the sixth lane from the left, most of the free probe has been eliminated from the bottom of the gel (red circle). This is an indication that the amount of protein added is likely too high for this particular probe, and virtually all the DNA has bound to the protein and shifted higher in the gel.

ED7 nuclear extract is expected to contain far less AP-2 protein than the extract from the older chick embryos based on our previous results (Bisgrove and Godbout, 1999). We therefore used differences between these two extracts as our primary criteria for determining whether predicted AP-2 binding sites were *bona fide* AP-2 binding sites. Most of the clones examined were uninteresting in that they showed no differences between the lanes containing ED7 and ED10 retina nuclear extract. As well, most clones displayed no significant changes in the presence of a 50X excess of cold AP-2 competitor DNA. However, clone 1213-13 [Figure 3.4.5(h)] and clone 0206-17 [Figure 3.4.5(p)] displayed bands, their positions denoted by the red arrowheads, that were present in the ED10 nuclear extract lane, but significantly reduced in the ED7 nuclear extract lane. These bands also showed a reduction in intensity when cold AP-2 oligonucleotide was added to the reaction; however, an unrelated cold competitor (SP-1) was incapable of diminishing these bands. Clone 1213-13 revealed no matches to previously characterized genes in a BLAST search, indicating that it may be a novel clone. Clone 0206-17 contains a stretch of nucleotides that aligns to a chicken genomic clone, WAG-93J15, as well as several chicken ESTs. Appendix A contains a summary of each clone that was analyzed in the EMSA reactions, including its sequence, the results of the BLAST searches, and the number of predicted AP-2 binding sites. Appendix B contains summaries of several clones that were not included in these reactions but were interesting nonetheless. These particular clones were excluded from EMSAs for the reason that either: (i) they showed significant alignments to ESTs throughout their entire length, or (ii) they showed alignments to the middle of a gene. Thus, these particular clones were not suitable for EMSA analyses, as they did not include promoter regions.

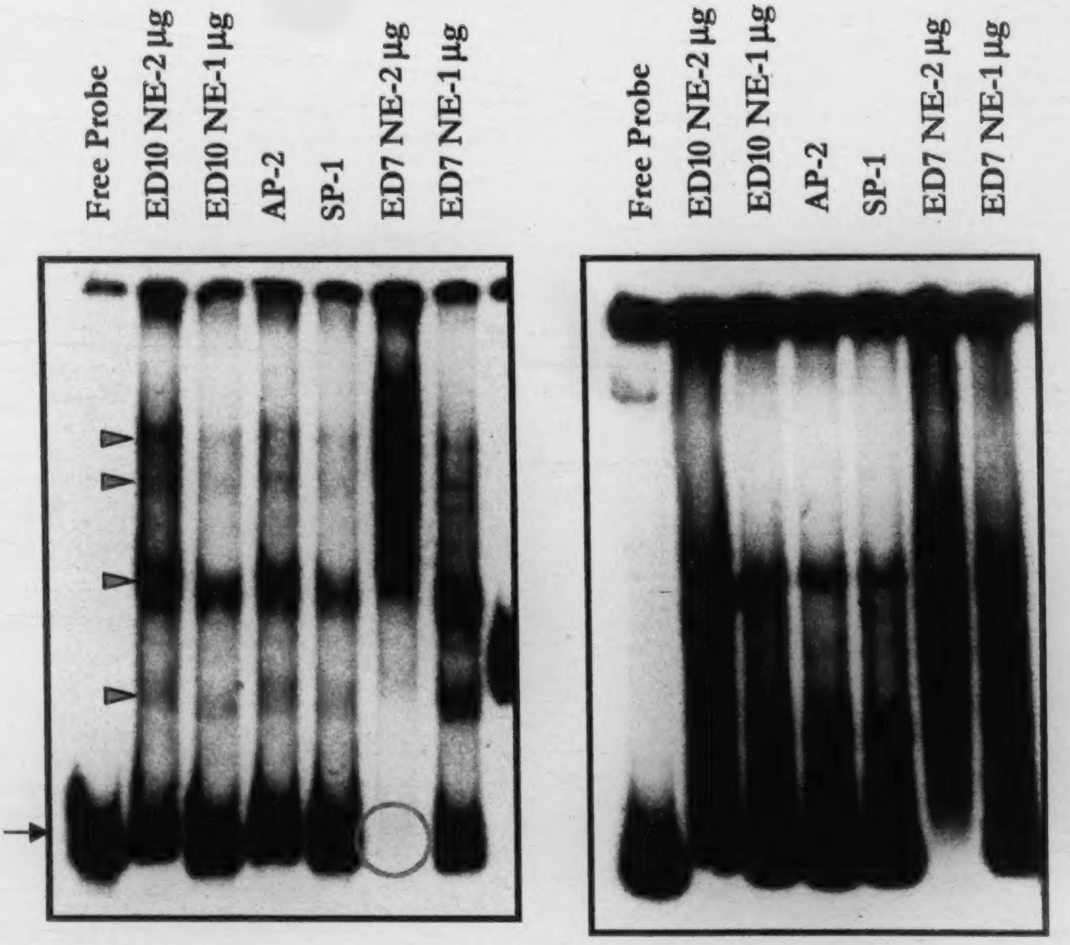

Figure 3.4.5(a): Autoradiographs of EMSA gels from clones 1205-3 (left) and 1205-4 (right). ED10 NE and ED7 NE refer to nuclear extract obtained from embryonic day 10 and embryonic day 7 chick retina tissue, respectively. The lanes labeled "AP-2" and "SP-1" contain a 50X excess of cold competitor oligonucleotide (Stratagene); 1 µg of ED10 nuclear extract was included in these reactions. No protein was added to the free probe lane. The blue arrowheads indicate protein:DNA complexes while the black arrow shows free DNA. No free DNA is evident in the sixth lane (red circle).

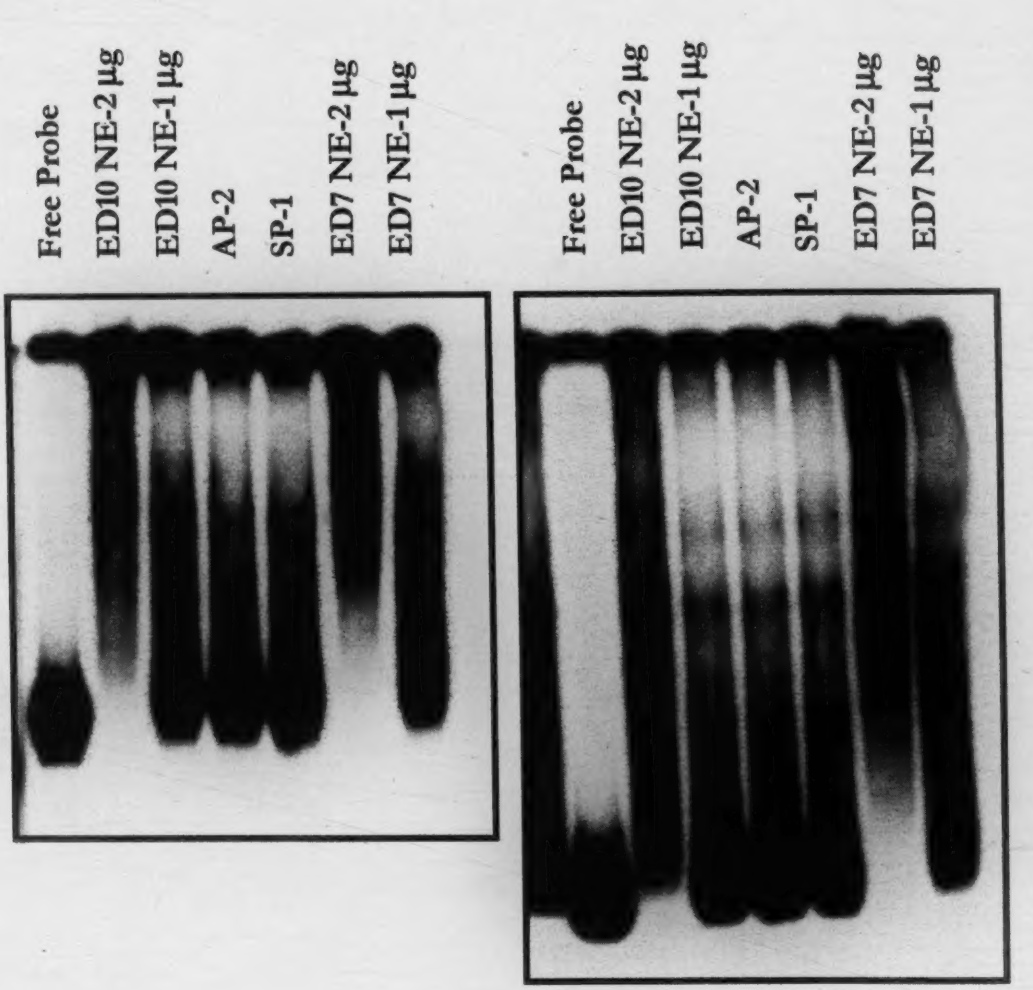

Figure 3.4.5(b): Autoradiographs of EMSA gels from clones 1205-22 (left) and 1205-24 (right).

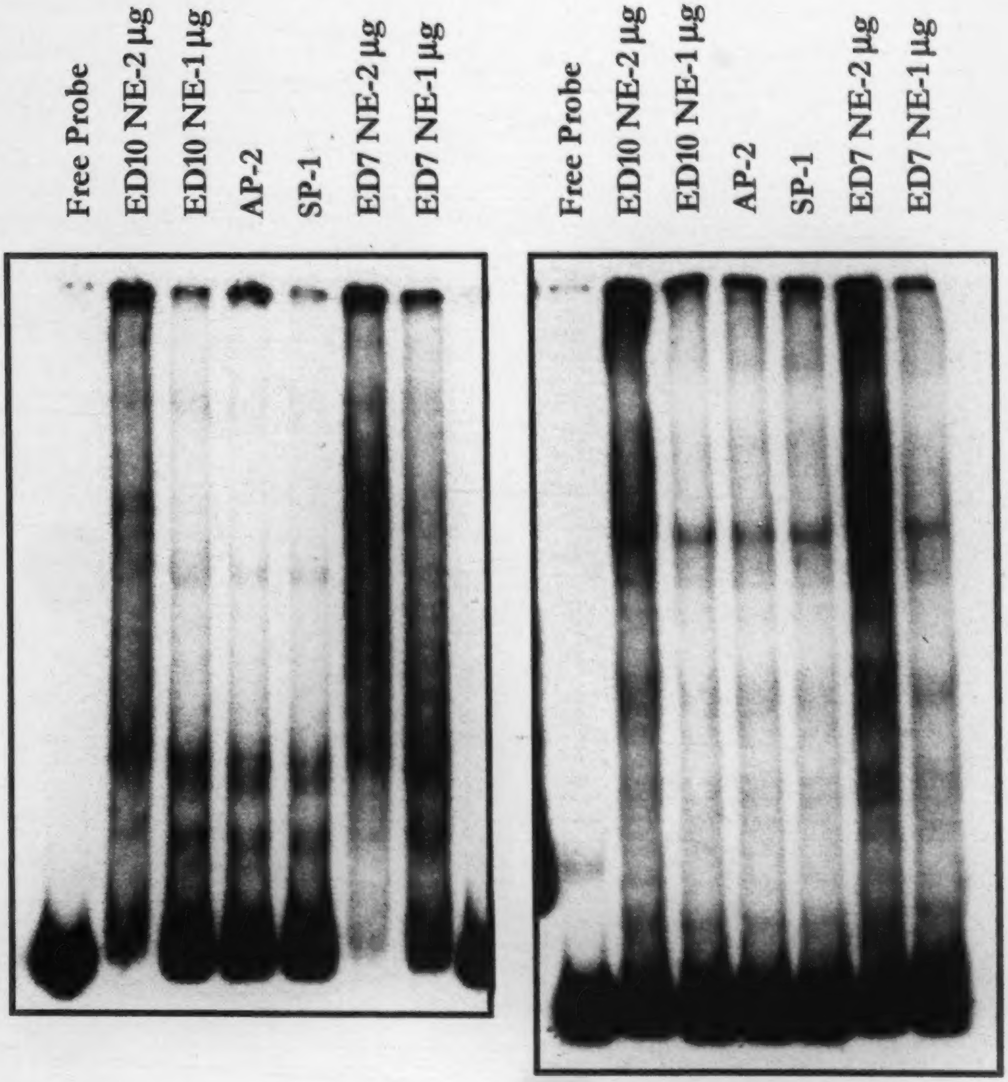

Figure 3.4.5(c): Autoradiographs of EMSA gels from clones 1210-1 (left) and 1210-5 (right).

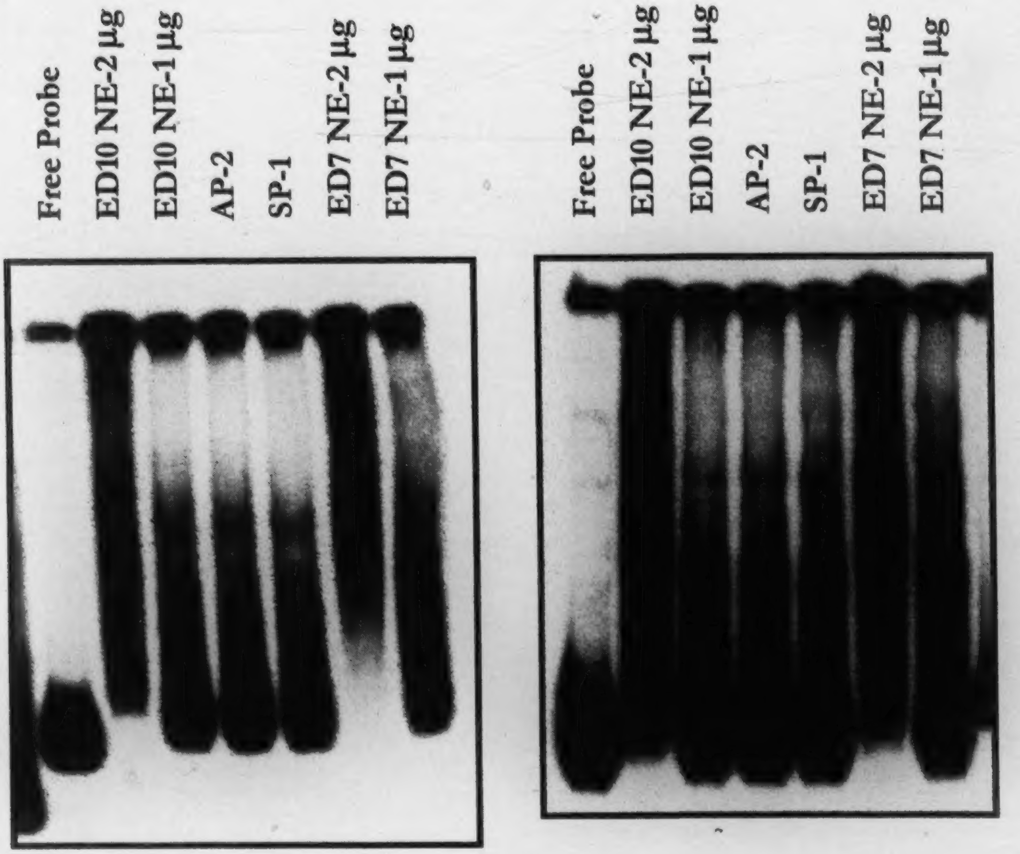

Figure 3.4.5(d): Autoradiographs of EMSA gels from clones 1210-9 and 1210-15 (left and right, respectively).

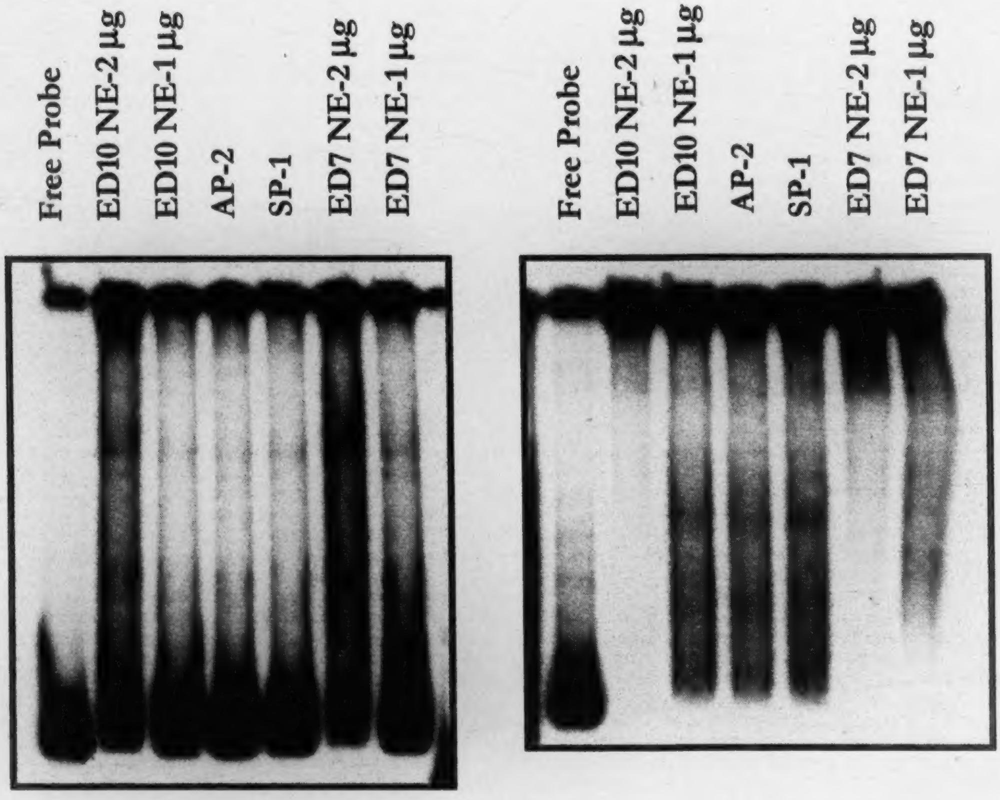

Figure 3.4.5(e): Autoradiographs of EMSA gels from clones 1210-17 (left) and 1210-18 (right).

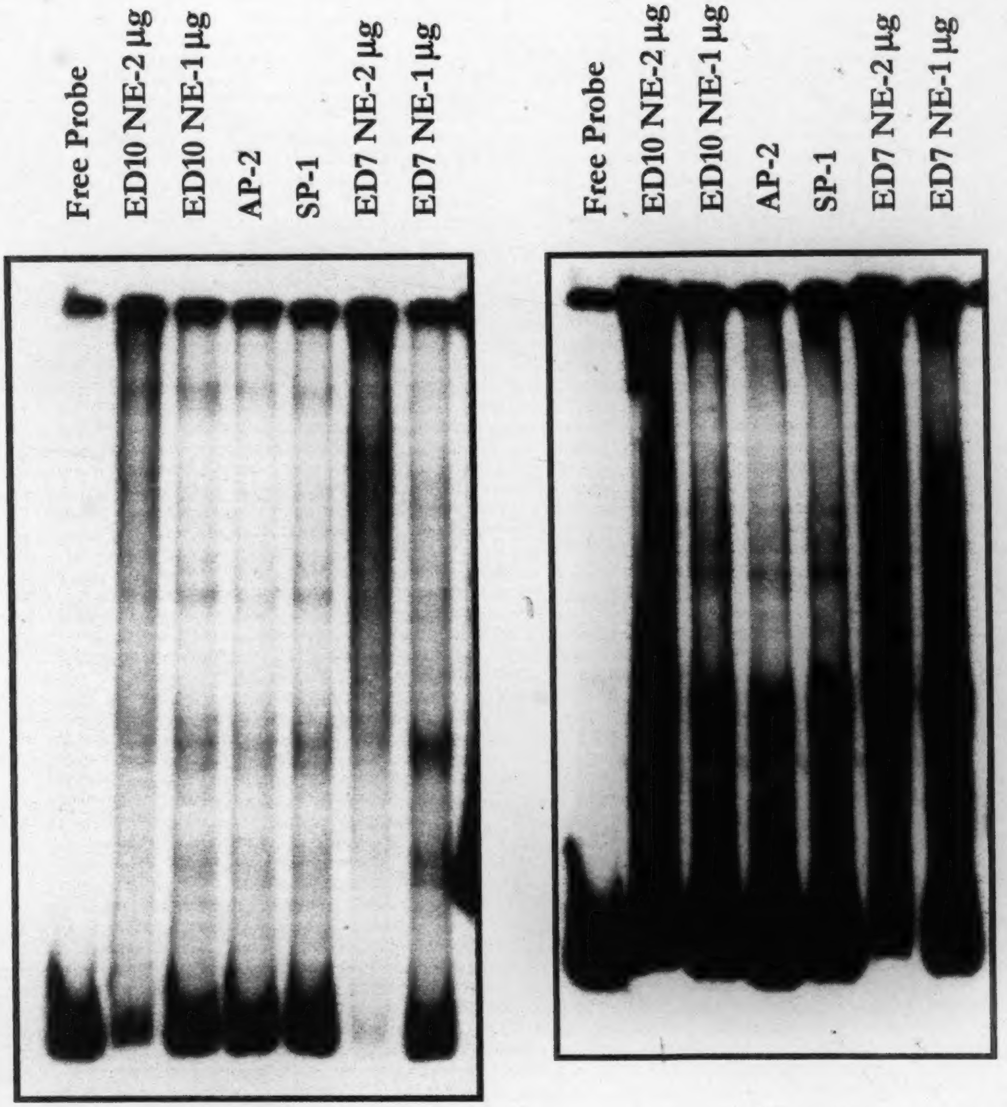

Figure 3.4.5(f): Autoradiographs of EMSA gels from clones 1210-22 and 1213-6 (left and right, respectively).

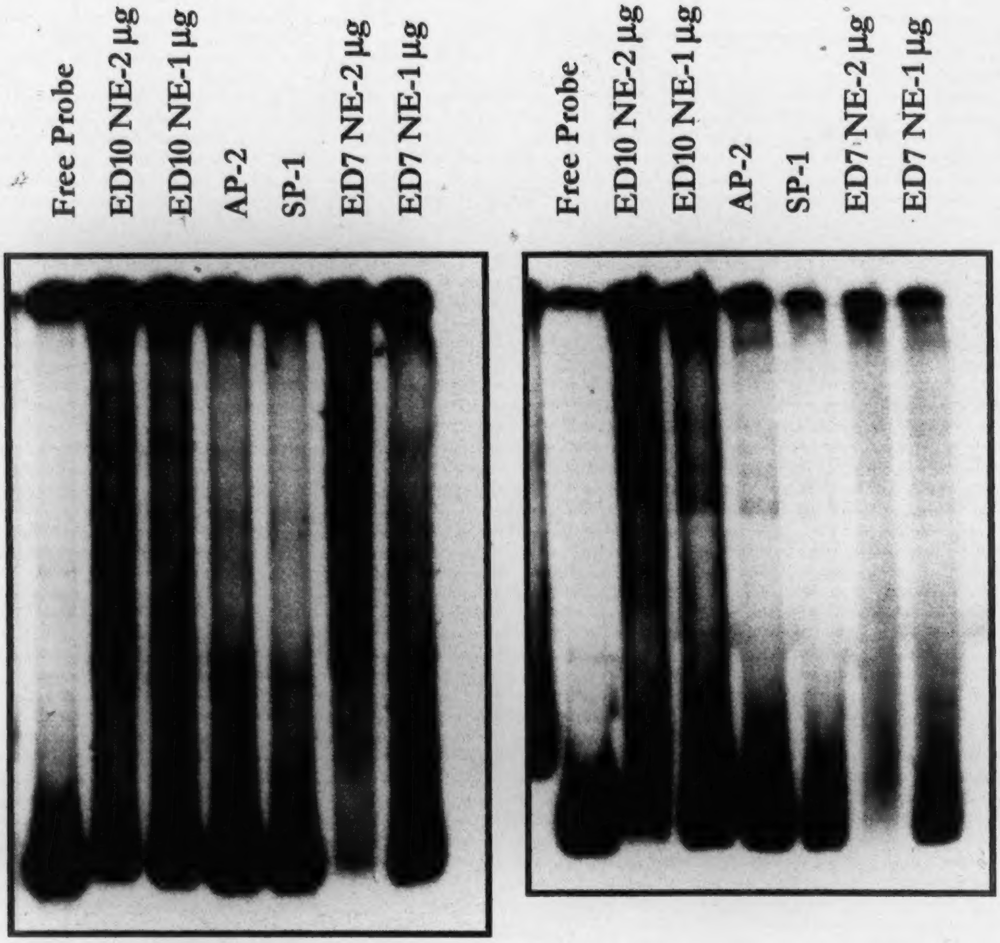

Figure 3.4.5(g): Autoradiographs of EMSA gels from clones 1213-3 (left) and 1213-4 (right).

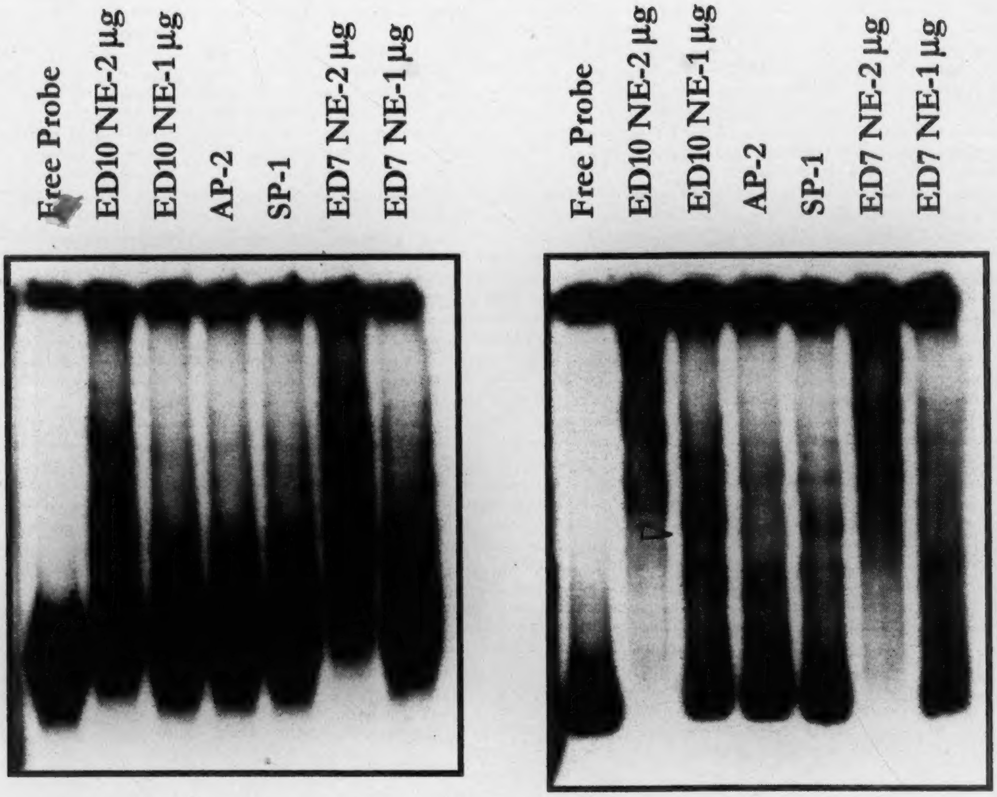

Figure 3.4.5(h): Autoradiographs of EMSA gels from clones 1213-9 (left) and 1213-13 (right). The position of a band on the 1213-13 gel is shown by the red arrowhead. This band, which is present in the ED10 nuclear extract, disappears upon the addition of cold AP-2 competitor DNA. This band is also absent in the ED7 nuclear extract.

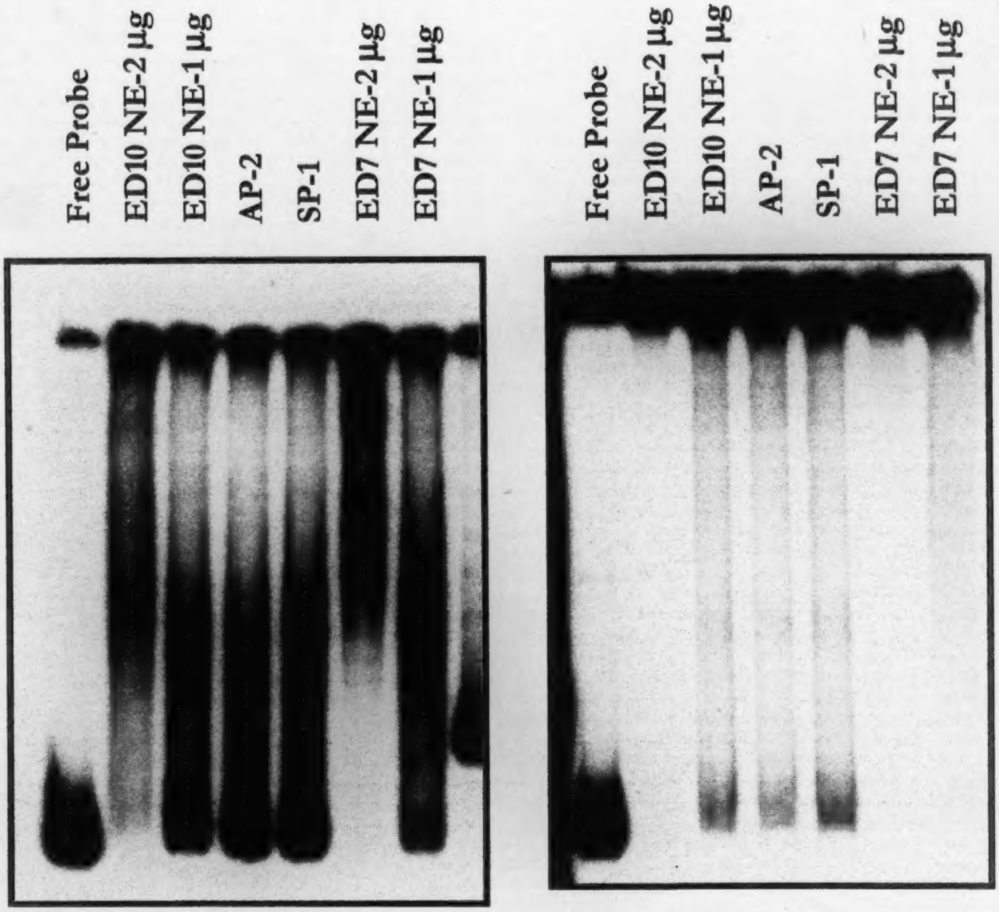

Figure 3.4.5(i): Autoradiographs of EMSA gels from clones 1213-2 and 1213-19 (left and right, respectively).

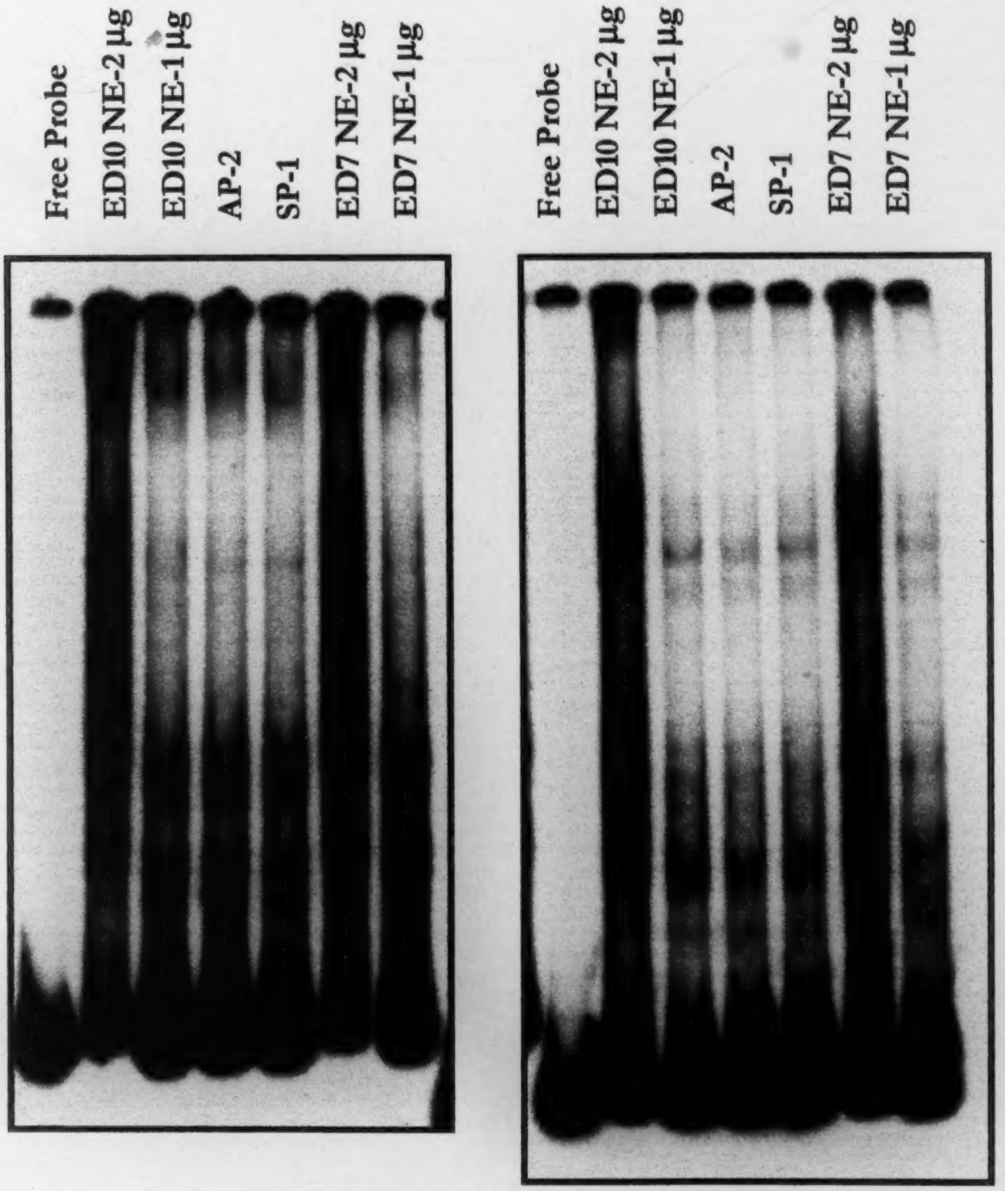

Figure 3.4.5(j): Autoradiographs of EMSA gels from clones 0115-2 (left) and 0120-4 (right).

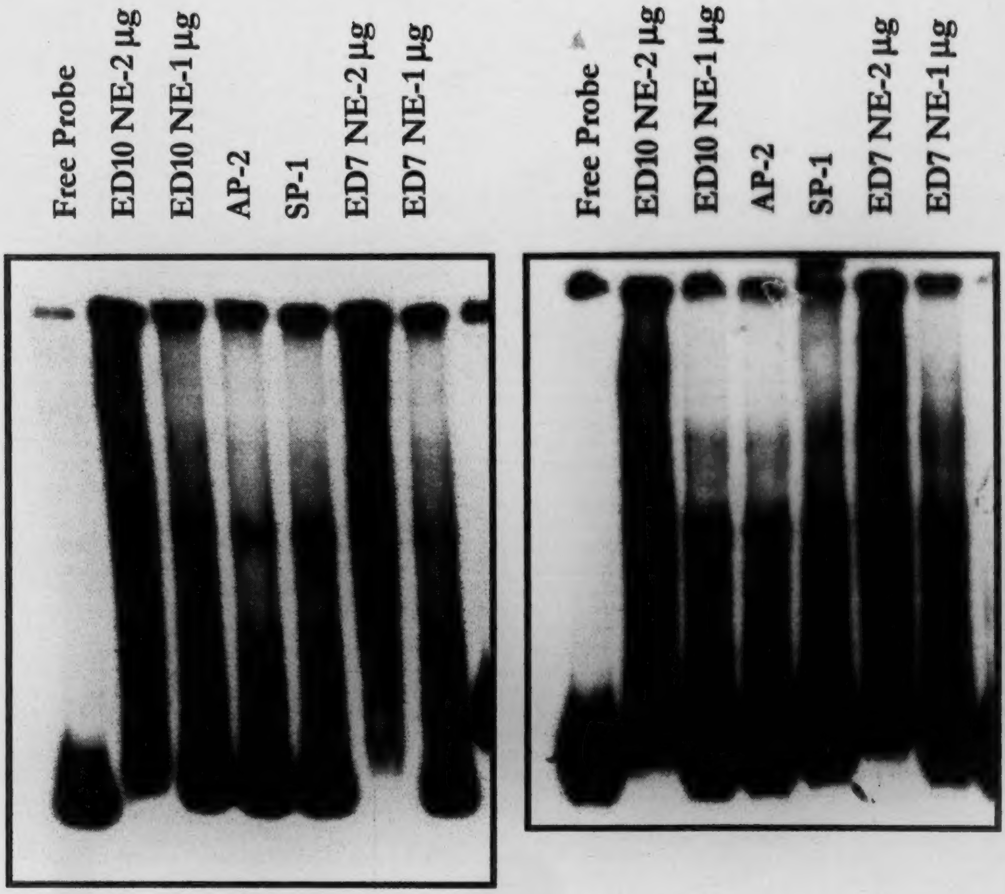

Figure 3.4.5(k): Autoradiographs of EMSA gels from clones 0115-4 (left) and 0115-7 (right).

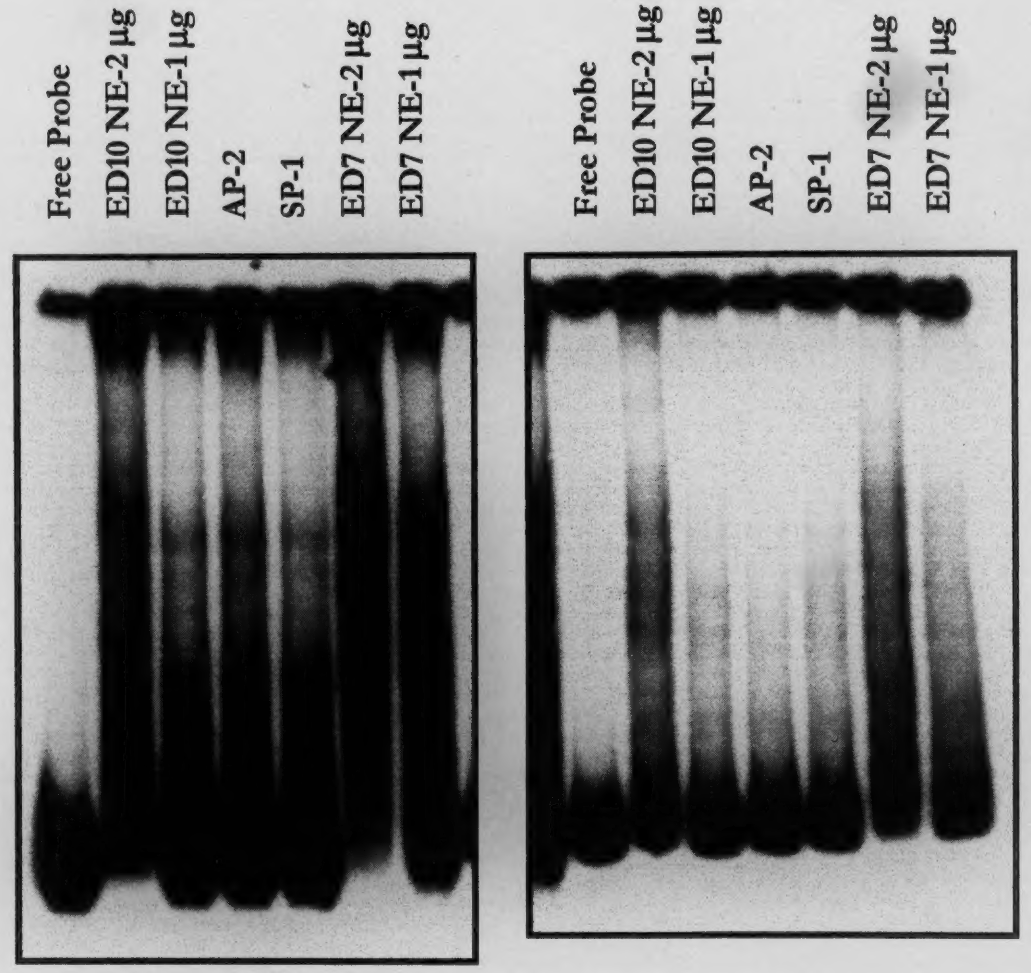

Figure 3.4.5(l): Autoradiographs of EMSA gels from clones 0115-17 (left) and 0115-18 (right).

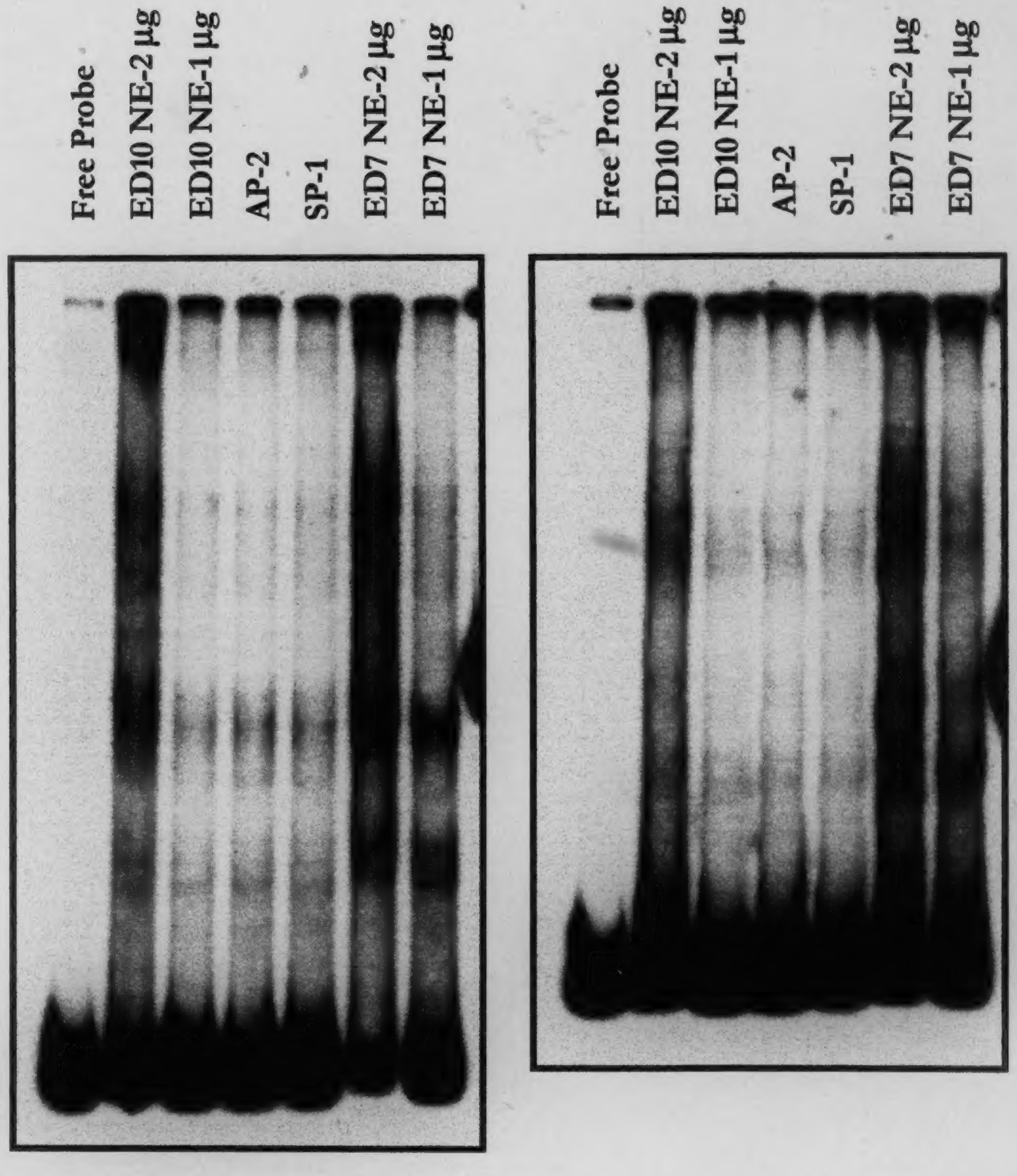

Figure 3.4.5(m): Autoradiographs of EMSA gels from clones 0120-5 (left) and 0120-19 (right).

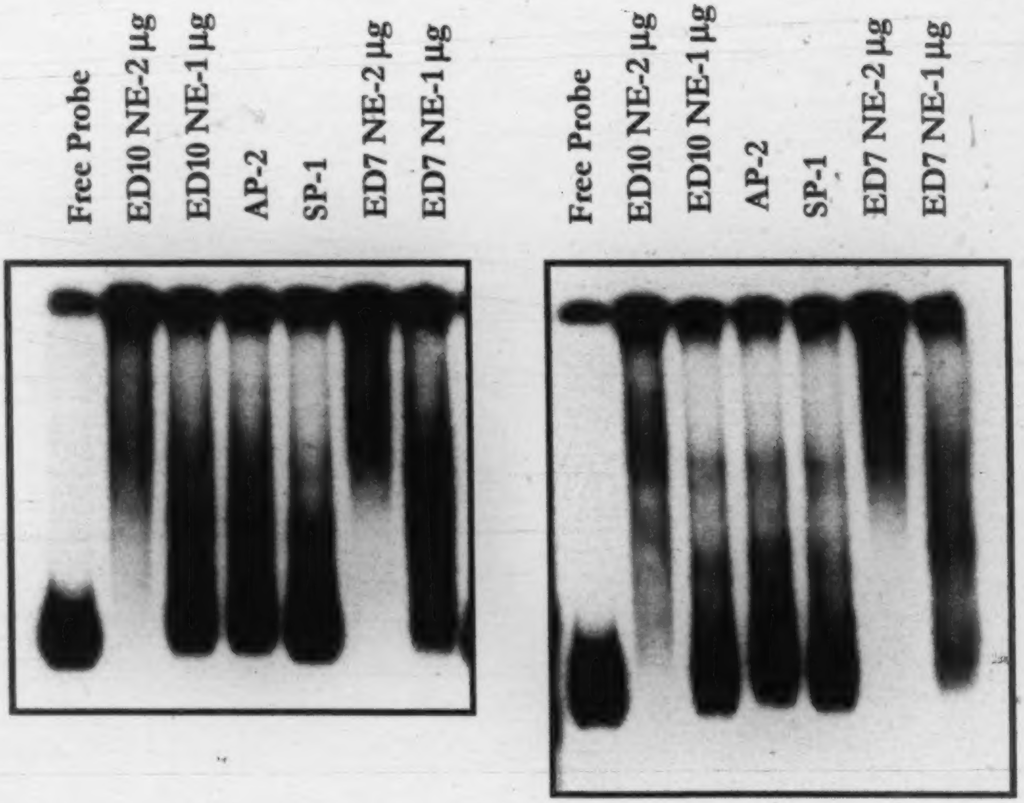

Figure 3.4.5(n): Autoradiographs of EMSA gels from clones 0120-11 and 0120-22 (left and right, respectively).

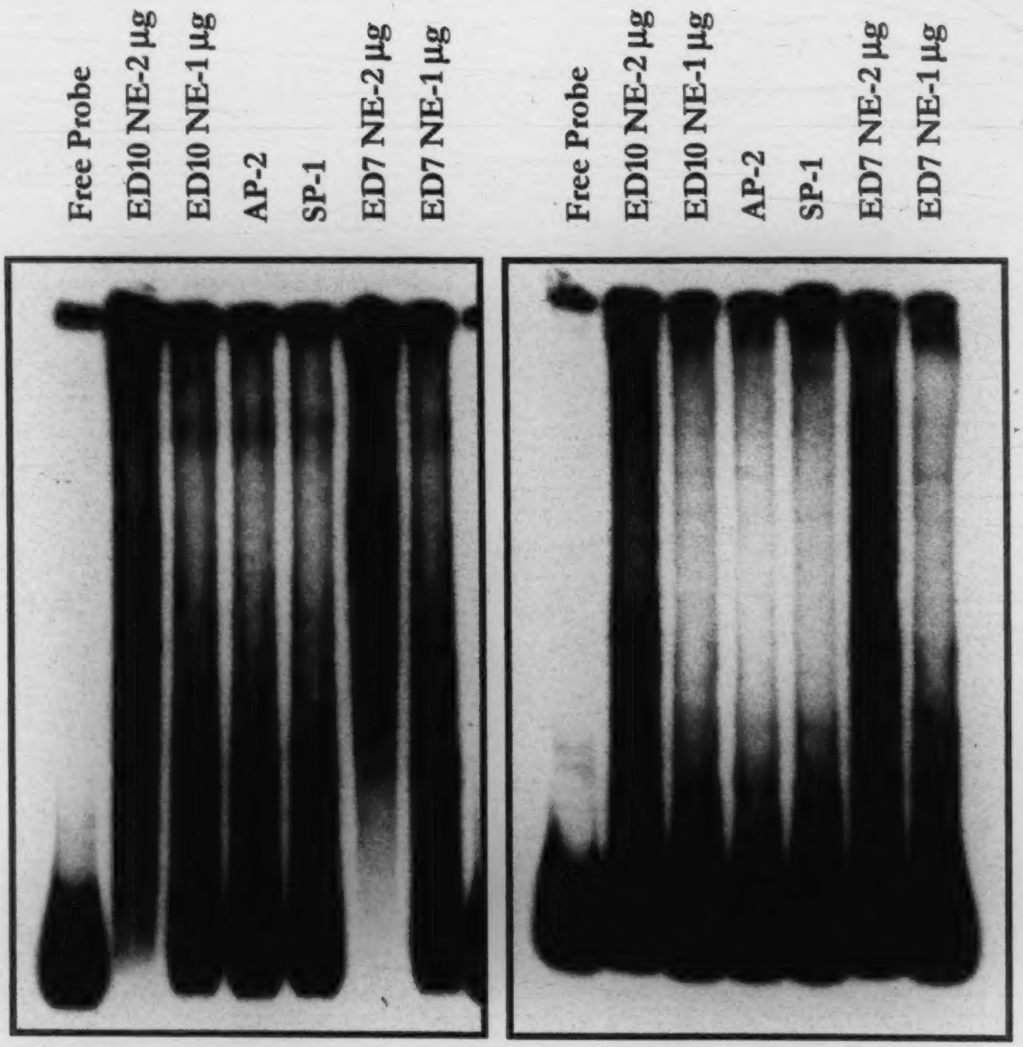

Figure 3.4.5(o): Autoradiographs of EMSA gels from clones 0115-9 (left) and 0120-2 (right).

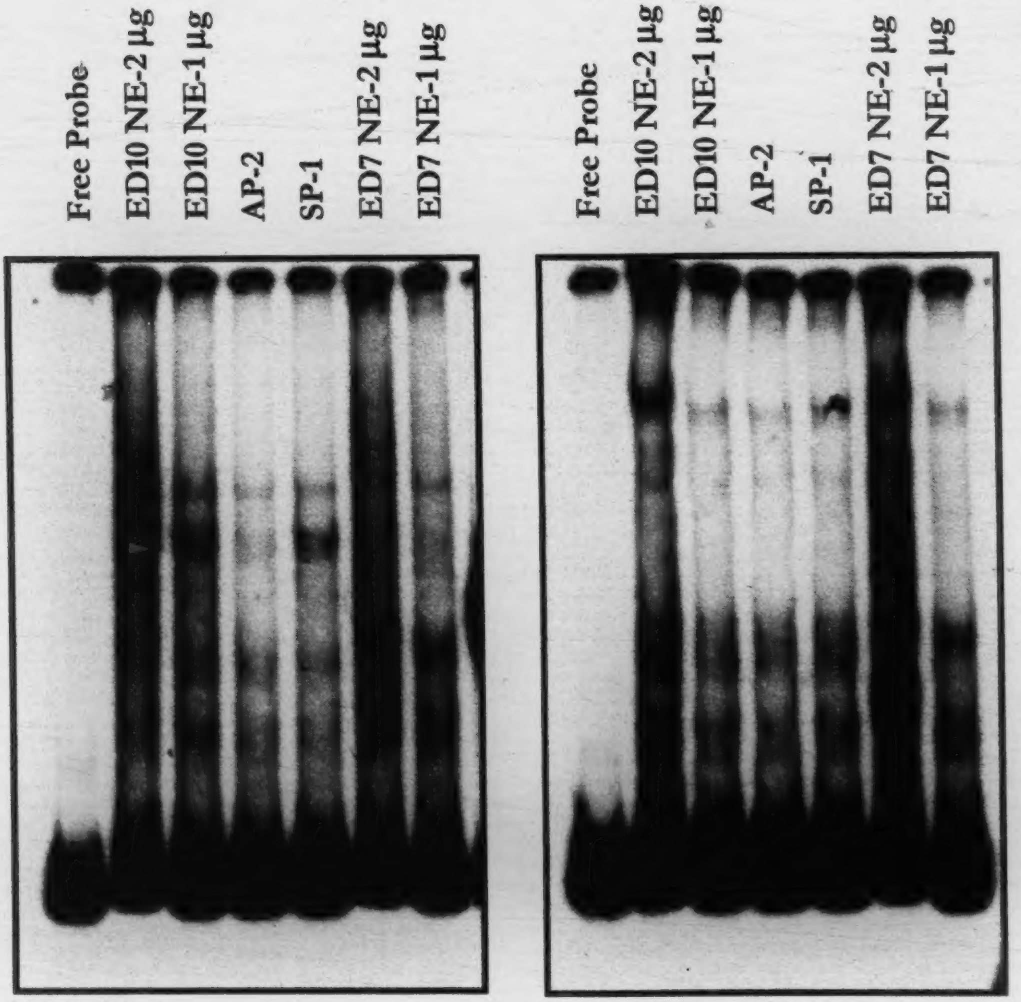

Figure 3.4.5(p): Autoradiographs of EMSA gels from clones 0206-17 (left) and 0206-23 (right). A red arrowhead on the 0206-17 gel shows a band that is present in the ED10 nuclear extract lane, competed out by an excess of cold AP-2 DNA, and absent in the ED7 nuclear extract lane.

CHAPTER FOUR

Results of the Retroviral-Mediated Introduction of Exogenous AP-2β into the Retinae of Developing Chick Embryos *in ovo*

The RCAS (Replication-Competent ASLV long terminal repeat (LTR) with a Splice acceptor) system of retroviral vectors (Hughes *et al.*, 1987) was used to introduce exogenous AP-2β protein into the retinae of developing chick embryos. In particular, we were interested in introducing AP-2β into the chick retina at an earlier stage of development than it is normally found. AP-2 expression begins in the retina at approximately ED7 (Bisgrove and Godbout, 1999), and the embryos were injected with a retroviral vector containing the AP-2β sequence at either ED2 or ED3. We hypothesized that exogenous expression of AP-2 would have an influence on cell differentiation, perhaps by forcing cells to develop prematurely along the horizontal and amacrine pathways. The results of these experiments are presented below.

Retroviral-mediated introduction of a gene into a chick embryo involves a number of steps. First, the coding sequence for the gene under study is cloned in frame into the RCAS/RCAN vectors in a two-step process. The sequence of interest is inserted into an adaptor plasmid, pSLAX, which contains rare restriction endonuclease sites (*i.e. Cla* I) that flank the insertion site [Figure 4.1.1(a)]. Importantly, a *Cla* I site is also present in the RCAS and RCAN plasmids. Thus, this intermediary step greatly facilitates cloning by generating cohesive ends on the insert in preparation for its ligation to the RCAS or RCAN vector DNA. Once the vector is prepared, the fertilized chicken egg is made ready by removing about 1.5 ml of albumin with a needle and syringe. Then a hole or "window" is cut in the top of the eggshell to expose the embryo, and DNA is injected into the structure of interest with a fine capillary tube. The addition of a small quantity of dye to the DNA helps to

ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC ATC GAT TCT AGA CCA
T3 primer——→ *Cla* I

CTG TGG CCA GGC GGT ACG TGG GAC GTG CAG CCG ACC ACC ATG GCC ATG ATT
***Nco* I**

ACG AAT TCG AGC TCG CCC GGG GAT CCT CTA GAG TCG ACC TGC AGC CCA AGC
***Eco*RI *Sma* I *Bam*HI *Pst* I *Hin*d III**

TTA TCG ATA CCG TCG ACC TCG AGG GGG GGC CCG GTA CCC AAT TCG CCC TAT
***Cla* I *Xho* I *Apa* I *Kpn* I**

AGT GAG TCG TAT T
←——T7 primer

Figure 4.1.1(a): Sequence of the *Cla* I insertion region of the pSLAX plasmid. pSLAX is a helper plasmid designed to facilitate cloning of a sequence of interest into the RCAS/RCAN plasmids. *Cla* I is a restriction endonuclease with a rare recognition sequence, and RCAS and RCAN have been designed such that they contain a single *Cla* I site. Thus, inserting a sequence of interest into pSLAX will generate *Cla* I ends on either side of the molecule, facilitating cloning into RCAS/RCAN. The sequence under study is inserted in frame with the ATG in the *Nco* I site to maintain correct translation of the protein. Figure adapted from Morgan and Fekete, 1996.

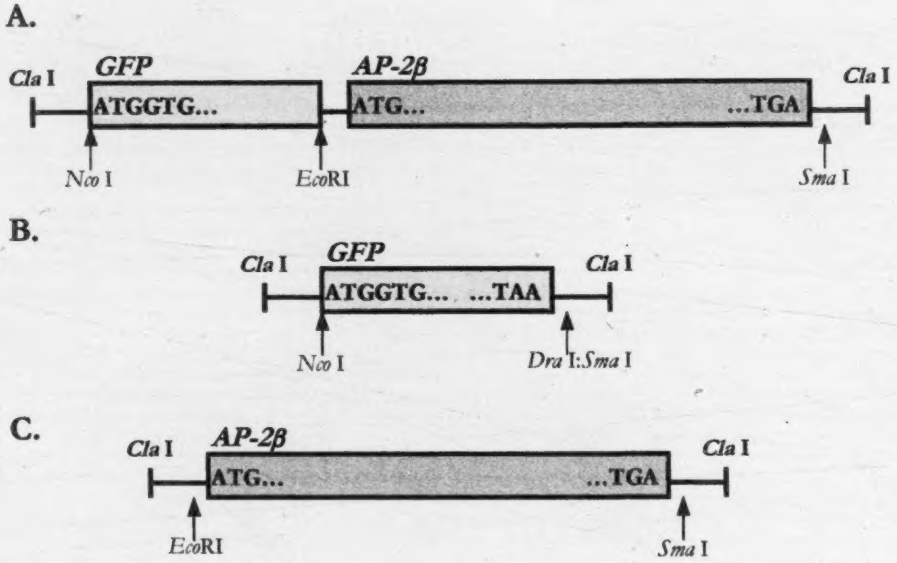

Figure 4.1.1(b): Diagrams of the three precursor constructs generated in pSLAX. Each construct was destined for cloning into the *Cla* I site in RCAS and RCAN. **A:** The construct encoding a GFP/AP-2β fusion protein. **B:** The construct encoding GFP. **C:** The construct encoding AP-2β. The restriction enzyme sites that play crucial roles in the building of these constructs are marked. Note that these diagrams are not to scale. The construction details are in Chapter Two.

visualize the site of injection. Electrodes are placed on each side of the injected structure and a charge is delivered, which creates temporary pores in cell membranes (Nakamura and Funahashi, 2001). Due to its negative charge, the DNA migrates toward the anode and is able to cross the cell membrane through the pores. Upon entering the cell, the vector DNA is transcribed to produce viral proteins and copies of the RNA genome for the synthesis and release of additional virus particles to infect neighbouring cells (Ogura, 2002). To permit this process to take place, the window is sealed shut with tape, and the egg is returned to the incubator where the embryo matures and the virus spreads to the dividing cells.

A map of the RCAS/RCAN plasmid, depicting the main features of this vector, is shown in Figure 4.1.2. These vectors are based upon the Rous sarcoma virus: the *src* region has been removed allowing insertion of another gene's sequence at this site, up to 2.4 kb in length (Morgan and Fekete, 1996). RCAS and RCAN differ by the presence of a splice acceptor site in RCAS, just upstream of the insertion site for exogenous DNA. Because RCAN lacks this site, it should be unable to generate the mRNA molecule that encodes the protein of interest, making it a negative control for infection. The products generated by alternative splicing in RCAS are shown in Figure 4.1.3. These mRNA species include two molecules that encode the viral machinery, and one molecule that corresponds to the gene of interest. This latter transcript makes up approximately 30% of the total splice products (*ibid*). All of the vectors were used with permission from Dr. Stephen Hughes (National Cancer Institute, Frederick MD).

4.1. Vector Construction and Testing

Six RCAS/RCAN constructs were made for *in ovo* retroviral infection studies; the intermediary pSLAX constructs are shown in Figure 4.1.1(b). A construct encoding a protein containing GFP fused to the N-terminus of AP-2β was designed so that the

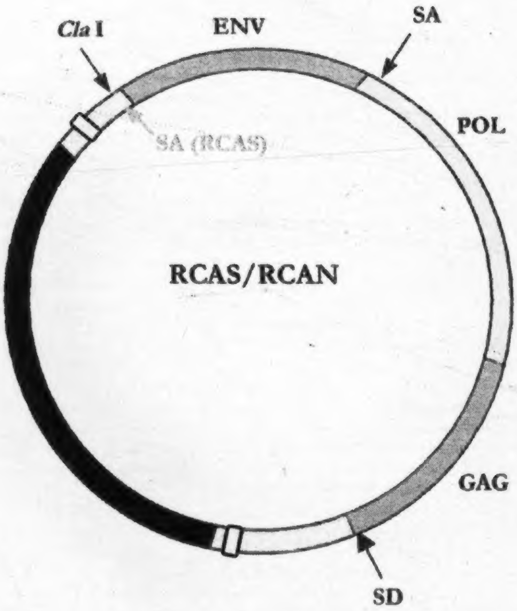

Figure 4.1.2: Map of the RCAS/RCAN proviral vector. The plasmid, ~11.5 kb in size, contains a unique *Cla* I restriction enzyme site for insertion of the sequence of interest into the vector. The vector contains sequence from *E. coli* (in black) as well as the viral genes *gag*, *pol*, and *env*. A splice donor (SD) and splice acceptors (SA) are marked. Note that the splice acceptor (in red) just upstream of the *Cla* I site is present only in RCAS. The two long terminal repeats (LTRs) are depicted by the yellow boxes. Adapted from Hughes *et al.*, 1987.

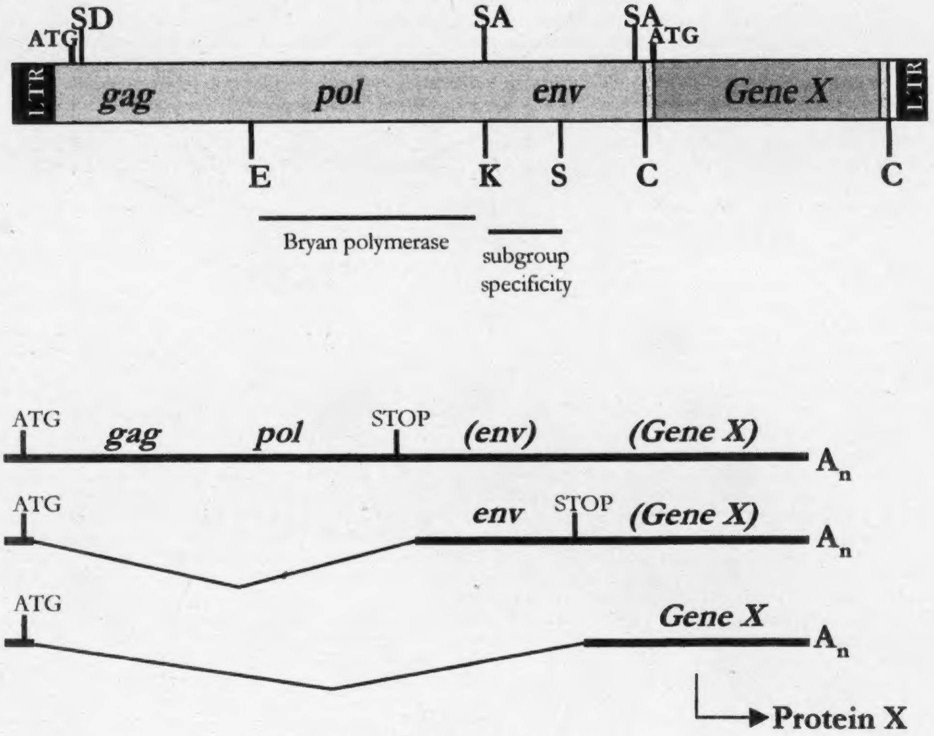

Figure 4.1.3: Diagram of a segment of the RCASBP (RCAS Bryan Polymerase) vector showing its main features and the three splice products. The *gag*, *pol*, and *env* viral genes are shown, as well as the long terminal repeats, translation start sites, splice donors and acceptors, and restriction enzyme sites. Sequence for the gene under study (*e.g.* Gene X) is inserted in frame into a unique *Cla* I site. Bryan polymerase is a high efficiency polymerase. The region marked subgroup specificity varies among vectors, allowing different chicken strains to be infected with the virus. SD: splice donor; SA: splice acceptor; LTR: long terminal repeat; ATG: translation start codon; E: *Eco*RI restriction endonuclease site; K: *Kpn* I site; S: *Sal* I site; C: *Cla* I site; A_n: poly A tail. Figure adapted from Fekete and Cepko, 1993.

infection of cells could be examined directly under fluorescent light, eliminating the need for other visualization methods such as *in situ* hybridization. The key difficulty with preparing this GFP/AP-2β fusion construct lay in the fact that the GFP coding region was to be inserted in frame into the *Nco* I site of the pSLAX adaptor plasmid, while the AP-2β sequence contained a number of *Nco* I sites and therefore could not be cut with this enzyme. Furthermore, a ligation of three fragments (GFP, AP-2β, and vector DNA) proved unsuccessful. Therefore, the following strategy was employed. The coding region of GFP was obtained by cutting the pEGFP-C1 vector (Clontech) with *Eco*R I and *Nco* I. This digestion removes the stop codon from GFP. The pSLAX adaptor vector was similarly digested with *Eco*R I and *Nco* I. The GFP insert and the pSLAX vector were ligated together and cut with both *Sma* I and *Eco*R I. Meanwhile, an available plasmid containing the AP-2β coding region (see Chapter 3, section 3.1) was cut to isolate the AP-2β insert. The first digest was performed with *Xho* I to liberate the 3' end of AP-2, which was then blunted with Klenow DNA polymerase. The DNA was extracted and then digested with *Eco*R I. The AP-2β fragment, with an *Eco*R I 5' end and a blunt 3' end, was ligated to the pSLAX/GFP fragment, also containing an *Eco*R I end and a blunt end.

The pSLAX vectors containing the AP-2β coding sequence or the GFP coding sequence alone were made as supplementary constructs in the event that the fusion protein did not function properly. These plasmids were intended for co-injection. For the former construct, the AP-2β fragment isolated above was cloned directly into the pSLAX vector, digested with enzymes to provide compatible ends. In the case of the latter construct, the GFP coding region including the stop codon was cloned into the pSLAX vector. The complete coding region of AP-2β is approximately 1400 bp in size, while the fragment encoding GFP is a little over 750 bp. Upon verification that the pSLAX constructs contained the correct inserts, the plasmids were cut with *Cla* I to liberate the inserts. A photograph of an agarose gel containing the products of these digests is shown in Figure 4.1.4. The inserts were ligated to *Cla* I-digested RCAS and RCAN vectors, and the correct orientation of each insert was confirmed by DNA sequencing (data not shown).

Prior to carrying out *in ovo* infections with the constructed plasmids, it was first necessary to test their ability to produce their corresponding proteins. This was achieved by transfecting cell cultures (*e.g.* d5 primary chick retinal cell cultures or chick embryo fibroblasts (CEFs)) with the plasmid DNA, harvesting the cells, and obtaining whole cell extracts. Western blots were made with aliquots of these cell extracts and immunostained to check for the presence of the exogenous protein of interest, either AP-2β or GFP. Results of the Western immunoblotting are shown in Figure 4.1.5(a-c). Blot A was incubated with anti-AP-2α C-18 antibody (Santa Cruz Biotechnology), which recognizes both AP-2α and AP-2β. The cells transfected with AP-2β RCAS had a faint band at ~50 kDa (denoted by the arrowhead), the correct size of AP-2. A similar size band was also detected in Dignam nuclear extracts prepared from ED10 chick retina (marked by the asterisk). The band in the AP-2β RCAS-transfected cells likely represents exogenous AP-2β, as no bands corresponding to AP-2 were detected in extract from cells transfected with an ALDH RCAS construct. A much stronger band was observed in the extract lane corresponding to cells transfected with the GFP/AP-2β fusion construct, and this band is approximately 80 kDa, close to the predicted size of this fusion protein.

Figure 4.1.5(b) is an immunoblot that has been incubated with an AP-2β antibody (Santa Cruz Biotechnology). The AP-2 band in the ED10 nuclear extract control is marked with an asterisk. A band similar in size is observed in the lane containing extract from cells transfected with AP-2β RCAS. In addition, a band that is likely the GFP/AP-2β fusion

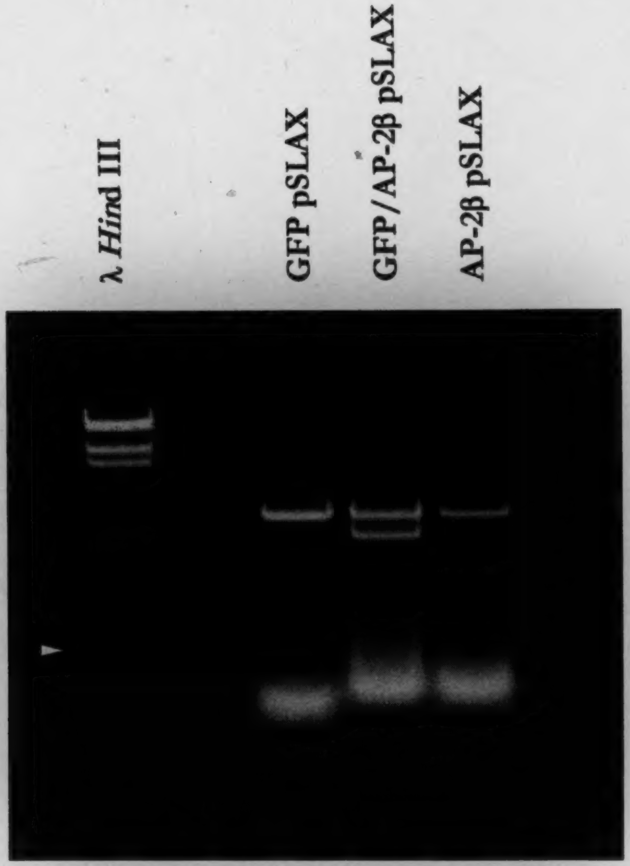

Figure 4.1.4: Restriction endonuclease digestion of the pSLAX intermediary constructs for the *in ovo* infection studies. Plasmid DNA from each preparation was digested with *Cla* I to liberate the inserts for analysis by agarose gel electrophoresis. The λ *Hind* III marker is shown on the left and the white arrowhead denotes the 560 bp band. The clones are from left to right, GFP pSLAX (~750 bp), GFP/AP-2β pSLAX (~2100 bp) and AP-2β pSLAX (~1400 bp). The pSLAX vector is ~3 kb in size. These fragments were purified and cloned into the RCAS and RCAN vectors to form the six constructs described in the text.

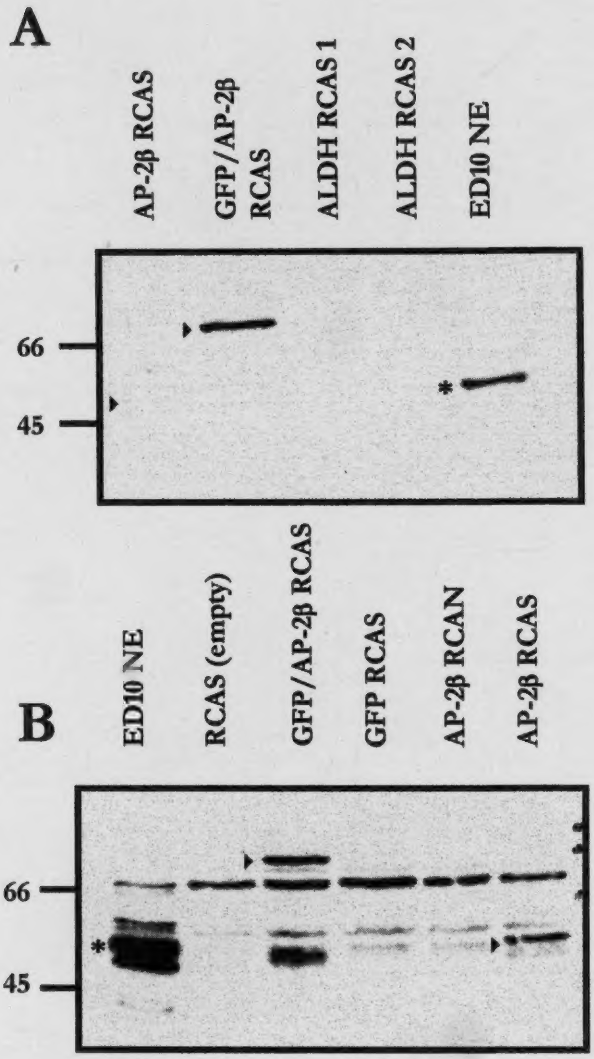

Figure 4.1.5(a,b): Western immunoblot results of whole cell extracts isolated following transfection of chick cell cultures with various RCAS/RCAN constructs. Size standards (in kDa) are indicated to the left of each blot. An aliquot of nuclear extract from ED10 chick retina cells was included on each blot as a positive control for immunostaining. The AP-2 band in each of these lanes is indicated by an asterisk. Arrowheads indicate bands that correspond to the protein being assayed. 50 μg of protein have been loaded in each lane. **A:** Blot of WCE from transfected d5 primary retinal cell cultures incubated with anti-AP-2α C-18 antibody (Santa Cruz Biotechnology. NB: This antibody is capable of recognizing both the α and β forms of AP-2.). Cell cultures were transfected with AP-2β RCAS, GFP/AP-2β RCAS, and ALDH RCAS (ALDH in this case was used as a negative control for AP-2β expression.). **B:** Blot of WCE from d5 primary retinal cell cultures transfected with the empty RCAS vector, GFP/AP-2β RCAS, GFP RCAS, AP-2β RCAN, and AP-2β RCAS. This blot was incubated with AP-2β antibody (C-20 Santa Cruz Biotechnology).

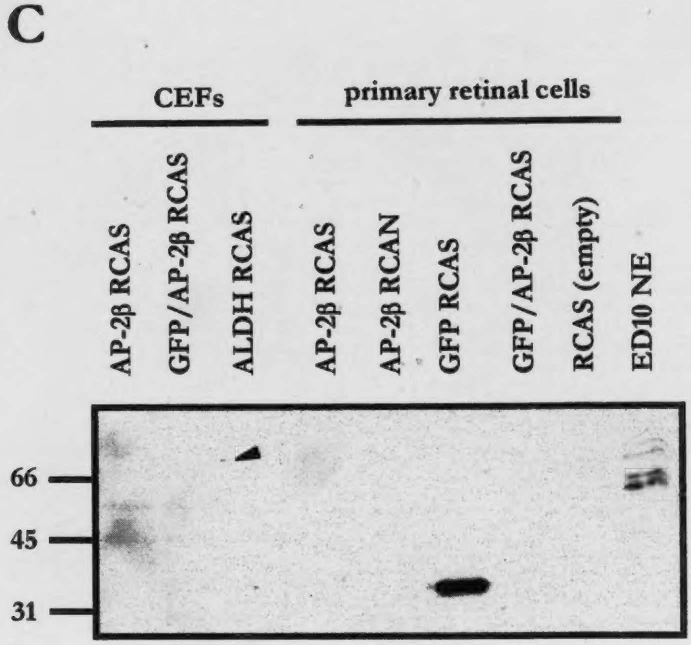

Figure 4.1.5(c): Western immunoblot results of whole cell extracts from chick cell cultures transfected with various RCAS/RCAN constructs. 50 μg of protein were loaded in each lane, and size standards (in kDa) are indicated to the left of the blot. A lane of ED10 nuclear extract (NE) was also included on the blot. The blot was incubated with anti-GFP antibody (Clontech) in order to detect exogenous GFP. The cells in this experiment included CEFs and d5 primary retinal cell cultures, and they were transfected with the constructs as indicated at the top of the blot. The arrowhead indicates a faint band in the GFP/AP-2 lane, possibly corresponding to the GFP/AP-2β fusion protein. Interestingly, anti-GFP antibody does not recognize the presence of a GFP/AP-2β fusion protein in the GFP/AP-2β RCAS primary retinal cell lane, although anti-AP-2 antibody is capable of recognizing this protein (see 4.1.5(a)).

protein is observed in the lane corresponding to the cells transfected with GFP/AP-2β RCAS. Western blot C was immunoblotted with anti-GFP antibody, and it revealed the presence of exogenous GFP in primary retinal cells transfected with GFP RCAS. A faint band (denoted by the arrowhead) may indicate the presence of the GFP/AP-2β fusion protein in the extract from the CEFs transfected with this RCAS GFP/AP-2β construct. Surprisingly, the GFP antibody does not detect a band in the lane containing extract from primary retinal cells that have been transfected with the GFP/AP-2β RCAS construct. The bands seen in the ED10 nuclear extract lane were unexpected and likely represent non-specific binding of GFP antibody to proteins found in this lane. Several western blots were also incubated with 3C2 antibody (obtained from Dr. Cairine Logan, University of Calgary, Calgary, AB), which recognizes the viral gag protein. However, the bands obtained on these blots were very faint and definitive conclusions about the presence of viral proteins in the cell extracts could not be drawn (data not shown).

4.2. *In ovo* Infections

Once it was established that protein was capable of being produced from the constructs, *in ovo* infections were undertaken. Fertilized eggs from White Leghorn chickens were incubated at 37°C until ready for injection at either Hamburger and Hamilton (HH) stage 11 or HH stage 18 (Hamburger and Hamilton, 1951). An embryo at HH stage 11 (40-45 hours old) is characterized by the presence of 13 somites and optic vesicles that are constricted at the bases, while a HH stage 18 embryo (65-69 hours) contains 30-36 somites and unpigmented eyes (the optic cup has fully formed by this time). These stages were chosen for injection, as they precede the stage of development at which AP-2 expression begins (ED7 or HH stage 31).

An early attempt at injecting the GFP/AP-2 RCAS construct into chick eyes is shown in Figure 4.2.1. In this particular case, a HH stage 18 embryo was injected with ~0.6 μg of the DNA construct, electroporated (25V, 50 msec pulse X5 with a 950 msec rest between each pulse), and sacrificed ~48 hours later. The embryo was enucleated, and the entire eye was examined under a laser confocal microscope. Fluorescent green cells were observed scattered throughout the eye's curved surface. Figure 4.2.1 shows a gallery of images: each photo represents a single picture of the GFP signal at a different angle of rotation. Unfortunately, it was difficult to tell if the cells observed here corresponded to retinal cells, as the retina could not be removed intact from the remainder of the eye.

Thus, subsequent injection attempts focused on introducing GFP/AP-2 RCAS into the retina proper. The results of one successful attempt are shown in Figure 4.2.2. The photographs consist of retinal tissue containing cells displaying the positive green fluorescent signal. The retina observed here is from an embryo that was injected at ED3 (HH stage 18) and sacrificed at ED7; the electroporation conditions were identical to those described above. In these photographs, the retina has been detached from the eye and flattened. Visualization of the GFP signal was achieved with a FITC filter on a Zeiss microscope and photos were taken with MetaMorph software (version 6.1r2). Successful retinal injections were also attained with HH stage 11 embryos (data not shown).

Following these experiments to confirm that retinal infections were feasible, additional experiments were set up. A dozen eyes positive for GFP signal were immediately preserved for sectioning to examine the effects of premature introduction of AP-2β on the distribution of retinal cells in the embryo. A GFP-positive eye from each of two electroporated embryos was sectioned using a cryostat. Rather than section the entire eye, 8 μm thick sections were taken at ~1 mm intervals. Several sections at each level were

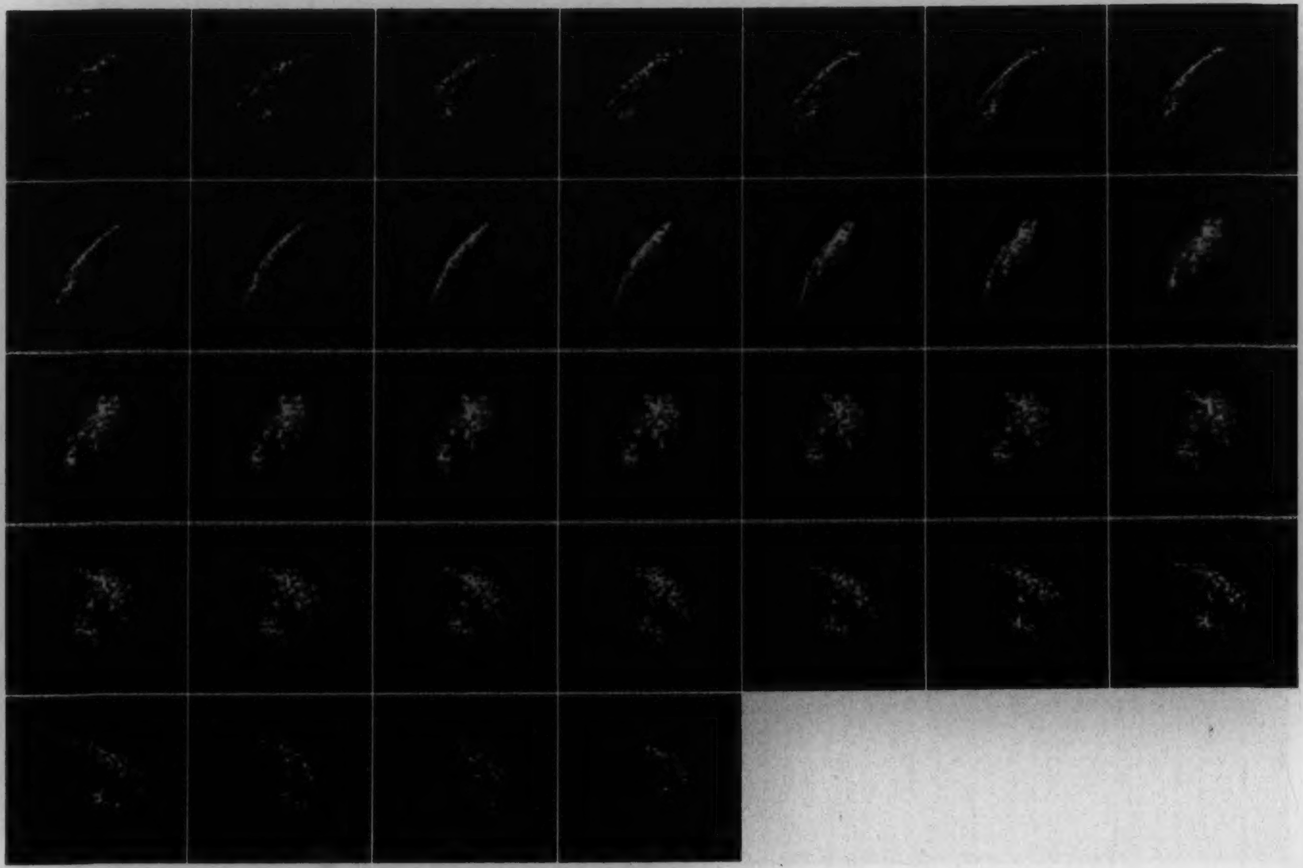

Figure 4.2.1: A projection gallery of a stack of images taken through the eye of a chick embryo following *in ovo* electroporation. The right eye of a HH stage 18 (ED3) embryo was microinjected with ~1 μl (~0.6 μg) of the RCAS GFP/AP-2 construct and electroporated (25V, 50 msec pulse, 5X, with a 950 msec pause between each pulse) with a BTX ECM 830 electroporator. The embryo was sacrificed ~48 hours post-injection. The images shown here represent a reconstruction of the GFP signal in the eye at different angles of rotation; these images were collected with a laser confocal microscope using a 488 nm argon laser and a long pass filter of 505 nm. The data are shown in gallery mode using the LSM 510 Zeiss program.

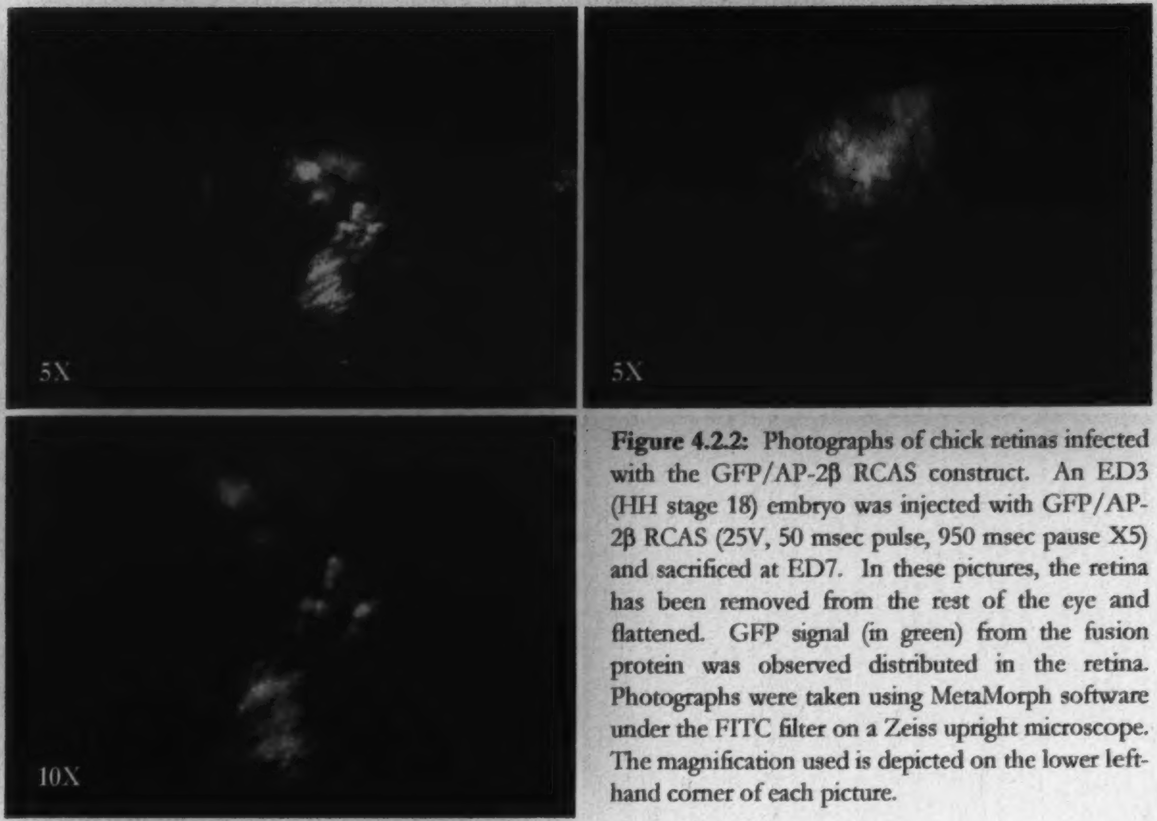

Figure 4.2.2: Photographs of chick retinas infected with the GFP/AP-2β RCAS construct. An ED3 (HH stage 18) embryo was injected with GFP/AP-2β RCAS (25V, 50 msec pulse, 950 msec pause X5) and sacrificed at ED7. In these pictures, the retina has been removed from the rest of the eye and flattened. GFP signal (in green) from the fusion protein was observed distributed in the retina. Photographs were taken using MetaMorph software under the FITC filter on a Zeiss upright microscope. The magnification used is depicted on the lower left-hand corner of each picture.

mounted to allow (i) visualization of the GFP signal, (ii) H&E staining, and (iii) staining with appropriate antibodies depending on the H&E results. The reasoning behind using this strategy was that it would significantly reduce the number of slides that needed to be created and analyzed, while hopefully including some of the affected area. Laith Dabbagh prepared the slides and completed the H&E staining (Department of Laboratory Medicine and Pathology, Cross Cancer Institute, Edmonton AB). Sections from one positive eyeball are shown in Figure 4.2.3. The sections on the left were stained in H&E, and the pictures were taken on an Axioskop 2 Plus (Zeiss) microscope. The layer of deep purple cells represents the neural retina while the pink area corresponds to the choroid layer. The photographs on the right were taken with fluorescent lamps and FITC filters. GFP positive cells are evident in the retina in these photographs. The sections of retinal cells in the H&E stained sections corresponding to the GFP/AP-2β infected cells appeared grossly similar to their normal, uninfected counterparts. The darkened portion of the retina in the stained section in Figure 4.2.3(b) is due to an artifact caused by some retina tissue folding upon itself. From these results, it does not appear that exogenous AP-2β has a significant effect on retinal cell development, at least for the duration that the virus was allowed to infect the embryo. It must be stressed that these results are very preliminary and final conclusions cannot yet be drawn until further experiments are performed. Although two eyes were selected for sectioning, only one showed GFP signal. Additional specimens need to be sectioned to determine the effect of misexpression of AP-2 on retinal development.

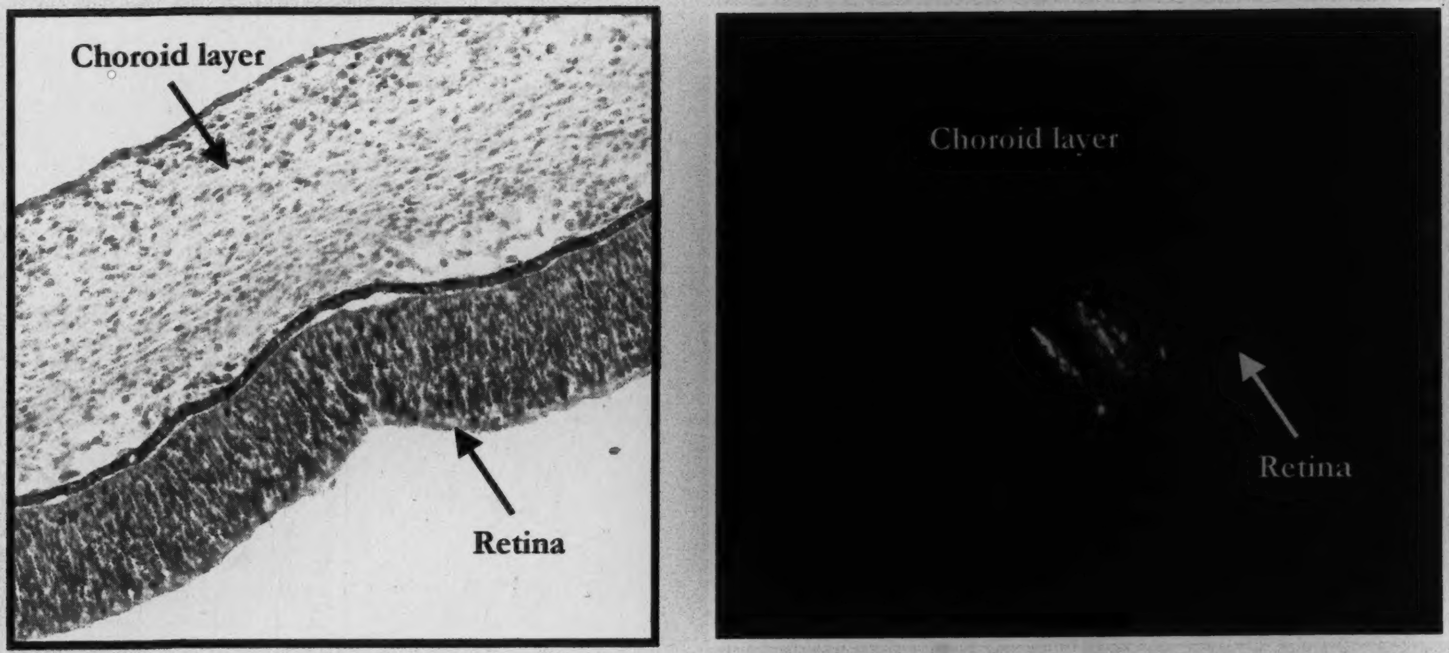

Figure 4.2.3(a): Adjacent sections of GFP/AP-2β positive chick retina. A HH stage 11 embryo was injected with GFP/AP-2β RCAS and electroporated (20V, 50 msec, 950 msec pause X5) and examined 6 days later. The eyeball was preserved by paraformaldehyde fixation for sectioning on a cryostat. **Left:** H&E staining of a section of chick retina. The neural retina consists of the layer of deep purple cells, while the pink area corresponds to the choroid layer. **Right:** Picture of an adjacent section taken under a fluorescent lamp. The GFP positive cells are in green. All sections are 8 μm thick.

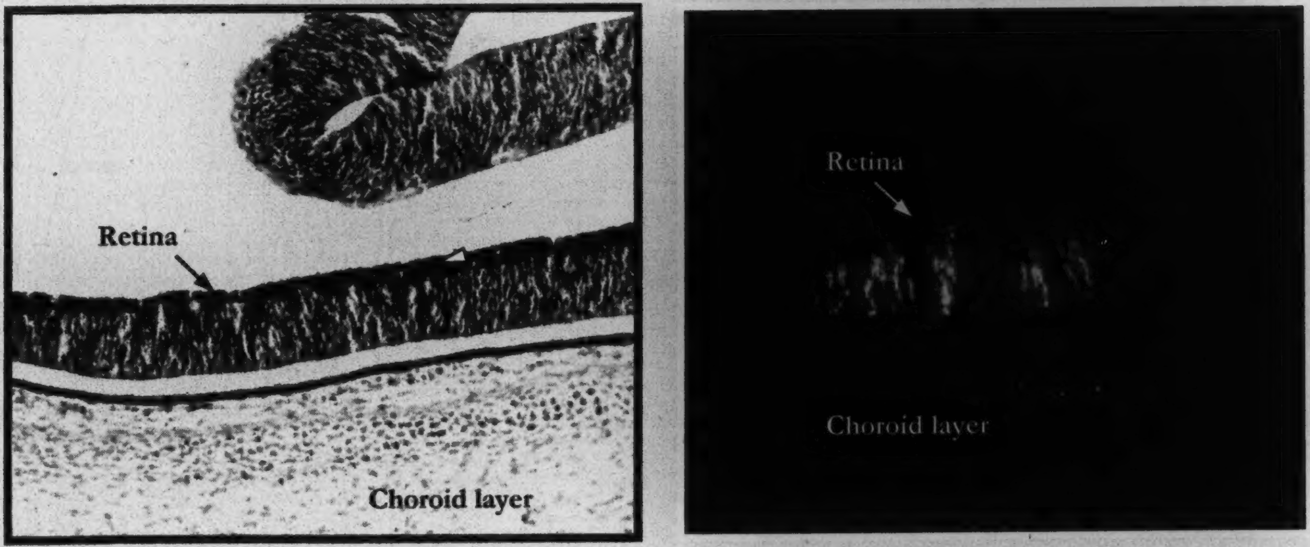

Figure 4.2.3(b): Adjacent sections of GFP/AP-2β positive chick retina from another area of the same eye depicted in 4.2.3(a). **Left:** H&E staining of a section of chick retina. The retina appears darker in the region denoted by the white arrowhead due to some tissue folding underneath itself during preparation of the slide. **Right:** Picture of an adjacent section taken under a fluorescent lamp showing the positive GFP signal.

CHAPTER FIVE

Discussion

Since its discovery by Mitchell *et al.* in 1987, numerous studies have focused on elucidating the properties and roles of the AP-2 transcription factor. For instance, investigators have established that this 52 kDa dimeric DNA binding protein consists of a family of four closely related members, AP-2α, -β, -γ, and -δ (Hilger-Eversheim *et al.*, 2000; Zhao *et al.*, 2001), that exhibit a high degree of conservation across several species (Whankhade *et al.*, 2000). The finer structural details of the AP-2 molecule have been defined, such as the helix-turn-helix motif that is critical for DNA binding and dimerization and the proline-rich transactivation domain (Williams and Tjian, 1991a and 1991b). In addition, AP-2α, -β, and -γ knockout mice demonstrate the significant role that AP-2 has in vertebrate development, apoptosis, and extra-embryonic tissue formation (Zhang *et al.*, 1996; Schorle *et al.*, 1996; Moser *et al.*, 1997; Werling and Schorle, 2002). Recently, AP-2 has been implicated in various cancers, including mammary carcinomas (McPherson *et al.*, 1997; Bosher *et al.*, 1995), malignant melanoma (Jean *et al.*, 1998; Huang *et al.*, 1998), prostate cancer (Ruiz *et al.*, 2001), and colorectal adenomas (Ropponen *et al.*, 2001), as well as a human genetic disorder, Char Syndrome (Satoda *et al.*, 2000). Studies have also revealed that a variety of genes are AP-2-regulated including *p21WAF/CIP*, transforming growth factor-α, estrogen receptor, *HER-2/neu*, and *MCAM/MUC18* (reviewed by Hilger-Evershiem *et al.*, 2000).

In our lab, Dwayne Bisgrove had previously performed experiments that demonstrated that AP-2 represses transcription of the *R-FABP* gene during development of the chick retina (Bisgrove *et al.*, 1997; Bisgrove and Godbout, 1999). Due to the large number of genes that AP-2 is known or postulated to regulate, we hypothesized that additional AP-2 targets exist in the retina. We have endeavored to find these unknown AP-2 target genes to advance the current knowledge of AP-2 with respect to retina development. As well, we were also interested in the effects that misexpression of AP-2 would have on the differentiation of retinal cells.

5.1. Evaluation of the Methods Used to Search for AP-2 Target Genes and Their Outcomes

5.1.1. *Differential Screening of an ED7 cDNA Library*

The first approach used to search for genes regulated by the AP-2 transcription factor in the developing chick retina involved differential screening of a cDNA library. To reiterate, duplicate plaque lifts of an ED7 chick retina cDNA library contained in the Lambda Zap II bacteriophage were probed with radiolabeled cDNA isolated from d5 primary chick retinal cell cultures transiently transfected with either an AP-2β expression construct or the corresponding antisense AP-2β construct. Notably, AP-2 expression begins in the chick retina around ED7 (Bisgrove and Godbout, 1999). Therefore, in the cultures transfected with the AP-2β sense construct, AP-2β would be introduced at an earlier stage of development than normal. Thus, the influences of AP-2β would hopefully manifest themselves in this cell population with respect to changes in the transcriptional activity of various genes, versus the other population, which lacked exogenous AP-2β expression. Bacteriophage plaques that showed a difference in signal intensity between the two filters after probing, representing the transcripts of genes presumably affected by AP-2β, were selected for secondary and tertiary rounds of screening. The DNA obtained from the interesting bacteriophage particles was then converted to plasmid form by *in vivo* excision for further characterization.

In total, seventeen clones were selected for further analysis, which included sequencing and Northern blotting. BLAST searches revealed five clones that did not match any previously characterized genes, while the others aligned to a variety of sequences including *G. gallus* mRNA for a ras-like protein, a 28S ribosomal RNA gene, a chicken T-cell receptor alpha-chain gene, and mitochondrial DNA. Mitochondrial DNA was omitted from further analyses, as it was considered an artifact, likely arising through contamination of the library during its preparation. Insert DNAs obtained from the remaining clones were labeled for Northern blot analyses. The Northern blots contained three lanes: the first consisted of ED7 mRNA, while the second and third were loaded with equal amounts of mRNA from the d5 cultures transfected with the AP-2β antisense or sense constructs, respectively. Thus, a variation in signal intensity between these latter two lanes, which differed only by the presence of exogenous AP-2β, would be an indication that the gene being analyzed was influenced at the RNA level by the presence of AP-2β.

The results of the Northern blot hybridizations were, for the most part, unremarkable. Many clones showed no difference between the last two lanes and thus were eliminated as candidate genes for AP-2 regulation. Others, despite multiple attempts at hybridization with freshly labeled probe, did not reveal any signal following autoradiography, even after long exposure times and washes of reduced stringency. Thus, data pertaining to their regulation status could not be obtained due to low RNA levels in the two cell cultures. A few clones, AP-2-10 (aligning with an unidentified gene), AP-2-2 (showing identity to a zinc finger protein), and AP-2-23 (aligning with the 28S ribosomal RNA gene) in particular, initially looked promising as AP-2 target gene candidates. Initial probing of the Northern blots with these cDNAs revealed differences between the two AP-2β-transfected lanes. However, subsequent attempts with other blots containing the same RNAs could not reproduce these differences. It should be noted, however, that the variations in signal intensity observed in the first trial were not very significant, in the order of two-fold or less.

In summary, this approach to searching for AP-2 target genes proved to be not very fruitful, as it yielded few concrete results. This technique was initially chosen as it seemed like a simple, straightforward approach that would produce at least a couple of genes for further study. The main drawback of this technique is that highly abundant transcripts, representing genes such as housekeeping genes, are more likely to show up in a screen such as this one than rare transcripts, which may be far more interesting. Thus another method was chosen to eliminate this bias towards these abundant transcripts.

5.1.2. *Suppression Subtractive Hybridization*

Following the results of the differential screening of the cDNA library, suppression subtractive hybridization was employed as a second method to search for novel target genes of AP-2 in the developing chick retina. In essence, SSH is a PCR based subtraction method that involves two populations of cells that differ by a single parameter, and it selects for transcripts that are unique to the population under the influence of that particular variable. Unlike differential screening, SSH is designed to enrich for sequences that are rare, due to the phenomenon of suppression PCR (Diatchenko *et al.*, 1996). During suppression PCR, DNA molecules containing short regions of homology at their ends (*i.e.* highly abundant cDNA sequences that form duplexes during the first round of hybridization, and therefore contain identical adaptors at their ends) are not amplified [see Figure 3.2.1(b)]. This is because in these situations, hybridization kinetics favour the formation of a closed, pan-like structure over the annealing of the primers to their complementary regions within the adaptor. SSH has the added advantage that it is less labour intensive than traditional subtraction methods and other techniques for isolating differentially expressed genes such as serial analysis of gene expression (SAGE). As well, several groups have reported success in finding differentially expressed transcripts with SSH. For example, Kuang *et al.* (1998) used SSH to identify genes that varied in expression between estrogen receptor (ER)-positive and ER-negative breast carcinoma cell lines. This group obtained 29 clones, 18 of which appeared to correspond to novel genes. Northern blot analyses revealed that the clones were expressed at least six times higher in the ER-positive cells compared to the ER-negative cells; in some instances, the differences observed in expression were a matter of the gene being on versus off in the two groups of cells.

The two populations used for SSH in our experiment were the same ones used in the differential screen of the cDNA library: *i.e.* mRNAs from d5 primary chick retinal cultures transiently transfected with sense and antisense AP-2β expression constructs, respectively. Thus, transcripts affected by AP-2β would be expected in the first population, designated the tester cDNA. The SSH procedure would remove or subtract sequences common to both populations by hybridization with the driver cDNA from the second group of cells, leaving only those rare sequences present in the tester to be amplified by PCR and retrieved for further characterization.

SSH was performed with the two populations of cDNA, and the final PCR products were cloned into pBluescript. 105 clones in total were picked from this library and insert DNA was obtained from each clone by electroelution. This insert DNA was used in the construction of slot blots, intended for a screening step that was recommended by the system's manufacturer to ensure that the clones obtained were, in fact, differentially expressed and did not result from background (*i.e.* cDNAs that were common to both populations). Duplicate slot blots, containing equal amounts of DNA, were each probed with mRNA from one of the two transfected retinal cultures. About 20% of the clones showed differences in intensity between the two blots. Like the putative AP-2-regulated clones obtained in the differential screen, DNA from these clones was then used as probes in Northern blot analyses. Again, only a few clones showed very slight differences between the lanes containing mRNAs from the cells transfected with sense and antisense AP-2β constructs. These clones included one with similarity to the guanine nucleotide exchange factor (GNEF) p532 and another with similarity to the S19 ribosomal protein.

The former clone would have been a very interesting finding, as it could be postulated that this protein has a role in controlling cell proliferation during retinal development by activating a second protein involved in a regulatory cascade that culminates in the transcription of certain genes, for instance. The slight variation between the lanes was not reproducible when this insert DNA was used as a probe on a second blot, though. It is also interesting that sequences corresponding to ribosomal elements were isolated in both the differential screening and SSH experiments. This may be a simple coincidence; however, there are studies that suggest that ribosomal proteins show temporal differences in expression (*e.g.* Lavery and Goyns, (2002), who showed that ribosomal protein S25 expression increases in the rat liver during ageing). It is reasonable that as cells mature, the amount of proteins that they synthesize changes, creating a demand for additional machinery that is responsible for the manufacture of new proteins. Notably, a recent report by Chen *et al.* describes the identification of several genes, including ribosomal protein S19, that are regulated by retinoic acid, the morphogen that induces expression of AP-2. These investigators generated RA-deficient mouse embryos, and mRNAs from these mice, as well as from normal embryos, were used in DD-PCR analysis to identify differentially expressed genes (Chen *et al.*, 1998).

Overall, the SSH approach to finding novel AP-2 target genes in the chick retina was not as productive as anticipated. For instance, out of the total number of clones recovered from SSH, only 20% appeared to actually be true positives from the results of the slot blot analyses. This number appears low, in light of other reports. Specifically, von Stein *et al.* (1997) performed SSH with a metastatic adenocarcinoma cell line and a non-metastatic population and found that 94% of randomly selected clones (68 out of 72) were, in fact, differentially expressed when analyzed by Northern blots.

One explanation for this seemingly poor enrichment of differentially expressed clones could be that there simply were not enough differences between the two populations used, and these differences were below the threshold that the system was capable of handling. In this case, the populations used were transiently transfected with AP-2 constructs. It is possible that only a small percentage of the cells in the cultures was transfected with the AP-2 expression vector, and thus had transcripts that were regulated by AP-2. If these *AP-2* transcripts were not highly abundant to begin with in a normal cell, the likelihood of isolating these transcripts from these transfected cells would have been low. An alternative approach to transfecting retinal cells with an AP-2 expression construct may have been to treat cell cultures with retinoic acid (RA), since AP-2 is known to be induced by RA. However, treatment of cells with this developmental morphogen would likely influence the expression of many genes, in addition to AP-2, thus complicating the search for AP-2 target genes. This approach would require much follow up work to verify that the isolated genes were indeed AP-2 targets. In retrospect, transfection of cells with an AP-2 expression construct was probably still the most feasible choice for inducing the expression of AP-2-regulated genes in the chick retina. Another possible flaw in the SSH procedure was that the secondary PCR following the hybridizations was omitted because one of the nested primers had a very high Tm. PCR using this primer set did not work, so the secondary PCR was performed with the same primer used in the initial amplification. Because the secondary PCR was designed to eliminate non-specfic amplification, this modification may have resulted in a higher background. Also, since SSH is based on PCR, it may be intrinsically prone to artifacts, as some sequences are more difficult to amplify than others based on their composition of base pairs.

5.1.3. *Isolation and Analysis of Candidate AP-2-Regulated Genes Identified from the Literature*

Three genes, choline acetyltransferase (*ChAT*), tyrosine hydroxylase (*TH*), and dopamine β-hydroxylase (*DBH*), were identified in searches of the literature as potential target genes of AP-2. The criteria used in the selection of these genes were first that they contained at least one AP-2 binding site in their promoters, and second, they were expressed in the retina, particularly in amacrine and/or horizontal cells. Kim *et al.* (2001), for example, identified domains in the human *TH* and *DBH* promoters that contained AP-2 sites and showed that exogenous AP-2 is capable of inducing activity from these promoters in HeLa and HepG2 cells. Similarly, Baskin *et al.* (1997) showed an interaction between an AP-2 binding site in the human *ChAT* gene and AP-2 through gel shift experiments with recombinant AP-2. As well, ChAT and TH are markers of amacrine cells (Dyer and Cepko, 2001), and DBH was shown by immunohistochemistry to localize to the retina of human and monkey eyes (Chen *et al.*, 1999).

This approach provided an alternative way to identify AP-2-regulated genes with relatively little effort. The fact that these human genes are reported to have AP-2 binding sites and are expressed in the retina does not necessarily mean that AP-2 is responsible for their expression in this particular tissue, and this relationship needs to be empirically determined. Partial mRNA sequences are published for all three chicken genes, and this information was used to design primers for RT-PCR to obtain sequences from each of these genes using mRNA from chick retina as a template. The recovered DNA fragments were then used to probe Northern blots, again containing a lane with ED7 mRNA, a lane with mRNA from d5 chick cells transfected with the antisense AP-2β construct, and a lane transfected with the sense construct. The results of the Northerns were inconclusive. For instance, the choline acetyltransferase and tyrosine hydroxylase probes showed virtually no signal on the northern blots. While the dopamine β-hydroxylase probe did show a slight signal, it was too weak to make any definitive conclusions regarding the status of this gene with respect to AP-2 regulation. It is possible that these genes are expressed at such a low level in the retina as to be undetectable by Northern blot analysis. Alternatively, these genes may not be expressed in the d5 chick retina, but they could be expressed later in development. The inclusion of mRNAs on the blot from later stages would have been informative in retrospect for this reason. The same potential problem may be arising in this analysis as discussed in the previous section: AP-2 may be influencing its effects in a small percentage of d5 cells due to the AP-2 expression vector being present in only a fraction of cells following transfection. AP-2 could also have no role in the regulation of these genes in the retina at this stage, if the necessary cofactors are not available, for example.

5.1.4. *Chromatin Immunoprecipitation*

Formaldehyde has been used as a reagent to cross-link proteins to DNA since the 1960s: these pioneering studies focused on investigating the distribution of histones along newly synthesized DNA (reviewed by Kuo and Allis, 1999). Chromatin immunoprecipitation is an extension of these early techniques, and recently, it has increased in popularity with regards to studies of transcription. ChIP is used primarily in two instances. The first involves confirming a suspected interaction between the promoter of a gene and the transcription factor of interest. In brief, primers are designed to surround the putative binding site(s), ChIP is performed with an antibody that recognizes the DNA binding protein, and the recovered DNA is analyzed by PCR. Following amplification, the presence of PCR product from the immunoprecipitated chromatin fraction is taken as evidence that the transcription factor is, in fact, binding to the promoter of the gene. Recently, ChIP has been employed to search for novel target genes of various transcription factors by cloning and analyzing the recovered DNA. For example, studies by Phelps and Dressler (1996), Weinmann *et al.* (2001) and Jishage *et al.* (2003) successfully used ChIP to look for new genes regulated by the Pax-2, E2F, and EWS/ATF-1 transcription factors, respectively. In each study, several clones were isolated that were then demonstrated to bind to and/or show regulation by the protein of interest in reporter assays. Searching for novel target genes is a more difficult application of ChIP than simply confirming a DNA:protein interaction, as it involves extra steps and requires more starting material. As well, no known target genes of a transcription factor may exist to act as a positive control for the ChIP reaction, such as in the case of the Pax-2 study just mentioned. No Pax-2-regulated genes had been previously identified prior to this investigation (Phelps and Dressler, 1996).

There are several benefits to using formaldehyde to cross-link proteins to DNA in a ChIP reaction. First, the cross-links generated by formaldehyde are easily reversible. Second, this chemical has high resolution in that it is capable of linking DNA and proteins that are in close proximity, or a distance of two Ångstroms apart. The main attraction to using ChIP is that unlike other techniques, it is performed *in vivo* versus *in vitro*, and thus should identify physiologically relevant targets of the transcription factor of interest. ChIP was chosen as an approach to search for target genes of AP-2 primarily for this reason. For instance, a potential drawback to using a technique such as SSH is that it can lead to the identification of indirect targets of a transcription factor such as AP-2. This would make analysis of the results of such an experiment much more difficult. Also, the possibility remains that by using cell cultures that express exogenous AP-2β prematurely, other factors are not yet present at this developmental stage, which are also required for the expression of AP-2-regulated genes.

A variety of challenges were encountered while implementing ChIP as a technique to search for novel AP-2 target genes in the chick retina. First and foremost was the availability of a good antibody. An antibody with a high affinity for the protein of interest is crucial to the success of a ChIP reaction. The chosen antibody must not only be capable of binding tightly to the target protein in an immunoprecipitation reaction and the subsequent washes, but it must also be able to bind to the protein when it is cross-linked to DNA. Occasionally, cross-linking masks the epitope that the antibody recognizes. For this reason, a polyclonal antibody is preferred over a monoclonal antibody as it increases the probability of successful recognition of the target epitopes (Aparicio, 1999). Thus, the antibody to be used should be tested extensively before implementing it in a ChIP reaction. A commercially available anti-AP-2 antibody, able to recognize both the α and β forms of AP-2 (Bisgrove and Godbout, 1999), proved to work very well in immunoprecipitations of both free and cross-linked protein. Unfortunately, when this stock became depleted, a second lot of the same antibody failed to work. It was necessary to test more antibodies from various sources until a suitable replacement was finally found. The chosen antibody, while still capable of recovering AP-2 from a cross-linked reaction, was less efficient than the first antibody. In order to circumvent this limitation, multiple immunoprecipitations were performed and the products were pooled together before analysis.

In addition, other details needed to be refined before proceeding with ChIP, such as suitable conditions for the sonication and cross-linking steps. For instance, the sonication parameters were adjusted by trial and error in order to generate fragments of a suitable size for cloning (*i.e.* under 1 kb) through purification and agarose gel analysis of the DNA fragments obtained in mock ChIP reactions. As well, the cross-linking conditions needed to be empirically determined: excessive cross-linking may result in a reduction in the fragment sizes obtained through sonication or interfere with DNA solubilization leading to a loss of material (Orlando *et al.*, 1997; Aparicio *et al.*, 2003). Inadequate cross-linking, on the other hand, may lead to fewer DNA fragments being recovered by ChIP for analysis. The extent of cross-linking was determined based on the principle that when proteins and DNA are loaded on a cesium chloride gradient and spun, free DNA migrates to the bottom of the tube, free protein remains near the top, and DNA:protein complexes end up near the middle. Therefore, by analyzing the DNA content of the various fractions by agarose gel electrophoresis, one can determine how much free DNA remains in comparison to the amount of DNA that is bound in complexes with proteins. We attempted to achieve cross-linking conditions that would allow as much DNA to become bound to protein in the cells as possible, yet permit the DNA to be sheared to the desired size during the sonication step.

ChIP was performed on ED10 chick retinae by compiling a variety of protocols (Weinmann *et al.*, 2001; Weinmann and Farnham, 2002; web page of Dr. P. J. Farnham, http://mcardle.oncology.wisc.edu/farnham/protocols/chips.html; Aparicio, 2003; Kuo and Allis, 1999; Orlando *et al.*, 1997; Upstate Biotechnology chromatin immunoprecipitation protocol, www.upstatebiotech.com). ED10 chick retinae were chosen for analysis as our lab had previously shown that AP-2 expression peaks at this stage (Bisgrove and Godbout, 1999). It was speculated that using material at this stage would increase the probability of finding AP-2-regulated candidate genes. After the ChIP reaction was completed, the recovered DNA fragments were cloned into pBluescript® with the aid of *Eco*RI linkers. In total, 59 plasmid DNA preparations were sequenced, and these data were then used in BLAST searches. Most of the clones remained unidentified following these analyses; however, this was not unexpected as there are far fewer chicken genomic DNA sequences in databases in comparison to other organisms. These sequences were then analyzed for putative AP-2 binding sites using several promoter analysis programs. Thirty-five clones contained at least one predicted AP-2 binding site. The clones that did not contain consensus AP-2 binding sites were considered to likely be false positives or contain as-yet uncharacterized AP-2 binding sites, and they were not analyzed further.

In order to establish if the predicted AP-2 binding sites within the ChIP clones were truly functional, electrophoretic mobility shift assays (EMSAs) were performed, following a procedure based on that reported by O'Brien *et al.* (1995), with labeled insert DNA from 32 of these clones as probes. The EMSAs were carried out such that a reaction containing Dignam nuclear extract from ED7 chick retinae (low AP-2 expression) was compared with a similar reaction containing nuclear extract from ED10 chick retinae (high AP-2 expression). As well, cold DNA (*i.e.* an AP-2 or SP-1 oligonucleotide) was included in the series of reactions to determine if the unlabeled AP-2 consensus oligonucleotide could compete with the labeled probes in binding to protein from the nuclear extracts. The EMSA gels revealed that the majority of the clones were not AP-2 targets by the observation that these DNA fragments showed no differences in protein binding between the ED7 and ED10 nuclear extract lanes. In addition, none of these DNA:protein complexes were eliminated upon the inclusion of a 50X molar excess of cold AP-2 DNA recognition sequence. However, two clones, 1213-13 and 0206-17, showed encouraging results. For instance, DNA:protein bands in these reactions were weaker in the ED7 lane when compared to the ED10 lane, and the signal from these bands was also diminished with the addition of the competitor AP-2 oligonucleotide. BLAST analyses of the sequences of these clones revealed that 1213-13 had no significant alignments to any known genes or ESTs, while 0206-17 contained a few hundred base pairs of sequence that aligned to various chicken cDNA clones of unknown origins. These clones definitely warrant further analysis, discussed below in section 5.3.

On the whole, ChIP proved to be a very challenging technique when applied to the search for AP-2 regulated genes in the chick retina. For instance, while the chick is a good model organism for retinal studies due to its ease of manipulation, the conservation of vertebrate eyes, and the abundance of ocular tissue at early embryological stages, the drawback to using the chick is the paucity of available genomic DNA sequences from this organism in databases (in comparison to the mouse or the human). The presence of additional chicken DNA sequences may have resulted in a more complete characterization of the clones obtained from ChIP.

Another difficulty encountered while implementing ChIP was the lack of genes known to be AP-2-regulated in the chick retina. With the exception of the *R-FABP* gene, no other gene to our knowledge has been shown to be a target of AP-2 in the chick retina. Several more of these genes would have been extremely useful in establishing positive controls for the ChIP reaction. In particular, PCR primers could have been designed to flank the AP-2 sites in the promoters of these genes to test if these sequences are recovered by ChIP. Such a control, in concert with a Western blot showing a successful immunoprecipitation would have given us added confidence that the ChIP reaction was working optimally. In fact, we did attempt to use the *R-FABP* gene in this capacity. Two primer sets were designed to flank the AP-2 binding sites in the *R-FABP* promoter. The first set failed to amplify sequence from chicken genomic DNA in preliminary experiments; the second pair was capable of producing a band of the correct size, albeit many other extraneous bands also resulted presumably due to false priming. However, this latter pair of primers was unable to amplify the sequence of interest from either the input chromatin or from the DNA recovered from ChIP. When this primer set was designed, the expected product had to be much longer than desired to avoid placing the primers in an extremely GC-rich region. It stands to reason that the longer the distance is between two ChIP PCR primers, the higher the likelihood the DNA will be sheared in the area between the primers during sonication. A future goal would be to design new primer sets for the *R-FABP* AP-2 binding site. A PCR was also attempted with a primer set designed to amplify sequence from the tyrosine hydroxylase promoter, even though it is not known whether or not this gene is actually regulated by AP-2 in the retina at ED10. As in the case of *R-FABP*, PCR product was not obtained from this gene following ChIP.

As previously stated, only two of the 32 genes analyzed by the EMSA gels appeared to actually bind to AP-2. Exhausting the first batch of anti-AP-2 antibody was certainly a disappointing setback, and it can be speculated that implementing a weaker antibody in the immunoprecipitation step may have resulted in a greater background, and therefore a higher number of false positives. Other studies do report obtaining false positives (*e.g.* Weinmann *et al.* (2001) observed that 36% of the clones analyzed in their experiment were false positives) so they may not be entirely attributable to the antibody used. For instance, inadequate washing of the protein-A beads (i.e. using wash buffers of too low a stringency, not performing enough washes) might be one reason for the false positives that arise during ChIP. Some groups include a second immunoprecipitation with the products recovered from ChIP to eliminate excess background (Weinmann and Farnham, 2002). The limitation with this approach is that the protein of interest must maintain its correct configuration under the salt conditions present in the elution buffer, so that the antibody continues to recognize it. An alternative way to improve the procedure might have been to purify the entire ChIP reaction on a cesium chloride gradient, dialyze the fraction containing the DNA:protein complexes, and then perform the immunoprecipitation on these products. This measure may have reduced the amount of excess free protein present in the reaction, which may compete with the cross-linked protein for binding to the antibody in the immunoprecipitation step.

A second explanation for the high number of apparent false positives observed could be that they did not arise through non-specific binding to the beads in the ChIP reaction, but are, in fact, actual AP-2 targets. Notably, an *Eco*RI digest was performed on the DNA recovered from ChIP in order to generate cohesive ends on linkers to facilitate cloning into a vector. This additional digestion step may have removed a portion of the immunoprecipitated DNA containing the AP-2 site, making it appear that the clone contained no consensus binding site. In support of this idea, some clones that were analyzed did not contain the three additional nucleotides at the *Eco*RI site that are expected to be present if the linker had been ligated to the ends of the ChIP DNA and then cut with this enzyme (The linker sequence is $^{5'}\text{CCG}\underline{\text{GAATTC}}\text{CGG}^{3'}$, so either CCG or CGG should be found immediately following or preceding the *Eco*RI site.). Some of the clones listed in Appendix B that were not included in the EMSA analyses due to an apparent lack of promoter sequence may fall into this category. To circumvent this problem, the DNA could have been treated with an *Eco*RI DNA methylase prior to adaptor ligation to protect the internal *Eco*RI recognition sites from being cut. This step was not included since it would involve an additional extraction to get rid of the excess enzyme, and the loss of material from this step would have been undesirable.

As well, genuine AP-2-regulated clones masquerading as false positives could have arisen in another way. AP-2 could be forming a complex with a second protein, which in turn, binds directly to the DNA. To illustrate, a recent study has reported that AP-2 binds to p53 at the p53 recognition site in the *p21* promoter, far away from any identified AP-2 binding sites. This study by McPherson *et al.* (2002) showed that this binding by AP-2 appears to augment the activity of p53. Thus, if AP-2 binds to another protein in this manner, the promoter sequence recovered in a ChIP reaction would not reveal the presence of an AP-2 binding site. In fact, there were several clones that lacked putative AP-2 regulatory sites, but contained p53 consensus binding sites.

The importance of using other experimental techniques to confirm the results of computer analyses to identify AP-2 binding sites is illustrated in the following two examples: clones 1215-4 and 0115-7 contained the sequences $^{5'}$GGGGCTGCC$^{3'}$ and $^{5'}$CCCGCTGGC$^{3'}$, respectively, which were predicted to be very good putative AP-2 binding sites. However, these clones did not appear to bind AP-2 in the EMSA analyses. Thus, one cannot make definitive conclusions about the regulatory status of a gene from promoter analyses alone. It was also interesting that the different programs did not always reach a consensus on their predictions. This is likely due to the fact that the programs draw upon various databases, which may differ in the completeness and accuracy of the transcription factor binding sequences that they contain. As well, two programs (AliBaba2.1 and Patch) that refer to the same database (TRANSFAC) often disagreed in their predictions. This is probably because the level of stringency used by the two programs differs significantly. Thus, the results of these promoter analysis programs have to be viewed with some skepticism. In addition, there seems to be some flexibility in the sequences that AP-2 is able to bind. For instance, the AP-2 consensus sequence is $^{5'}$GCCNNNGGC$^{3'}$; however other motifs have been reported to bind AP-2 such as $^{5'}$GCCNNNNGGC$^{3'}$, $^{5'}$GCCNNNGGG$^{3'}$, or $^{5'}$CCCCAGGC$^{3'}$ (reviewed in Hilger-Eversheim *et al.*, 2000). This brings up the possibility that not all AP-2 binding sites have been described yet. It is possible that performing EMSAs on the sequences containing no predicted AP-2 sites will reveal instances of AP-2 binding.

One concern with using EMSA gels to confirm the computer promoter analyses is that the size of the probes used in some cases was pushing the reaction to the limits of its capabilities. For example, DNA fragments of 50-400 bp are reported to work well in EMSA reactions (Laniel *et al.*, 2001); however, a few of our DNA fragments exceeded this length. The autoradiograph of clone 1213-19 shows that most of the labeled DNA probe is likely bound to protein in the nuclear extracts, forming huge complexes that are unable to enter the gel (thereby generating a strong signal near the wells and no signal in the lanes). Cutting the probes into smaller fragments and testing the individual pieces, will be required for some of the larger probes. To further document bona fide AP-2 binding sites, an attempt was made to insert the AP-2β coding sequence in the pGEX-4T2 expression vector with the goal of expressing recombinant AP-2β protein for the EMSA analyses. The complete coding region of AP-2β was generated by PCR with *Taq* polymerase, containing PFU to remove the one base overhangs, so it could be inserted into the *Sma* I blunt site of pGEX-4T2. Recombinant protein was indeed expressed from this vector, but the product appeared slightly smaller in size than expected on a protein gel (data not shown). The inherent risk in using PCR to amplify a sequence for cloning in this manner is that a replication error may be propagated, which could generate a truncated protein if this mistake creates a premature stop codon. A future goal will be to generate full-length AP-2 recombinant protein and use this protein in EMSA reactions.

5.2. Retroviral-Mediated Introduction of Exogenous AP-2β into Chick Embryos

We were also interested in investigating effects of premature AP-2 expression on the developing chick retina. We had speculated that inappropriate expression of AP-2 would have a significant impact on retinal development, particularly on the differentiation of horizontal and amacrine cells. AP-2 is first expressed when retinal precursor cells become committed to these two pathways. In order to express exogenous AP-2β in the chick retina, we employed the Rous Sarcoma virus derived RCAS system of retroviral vectors, coupled with electroporation, to introduce viral sequence containing the AP-2β coding region into chick retinal cells. Upon entry of the provirus into retinal cells and its integration into the host DNA, new viral particles would be produced and released to infect other neighboring cells, thus propagating expression of the AP-2β protein.

Injection of the proviral DNA into a structure of interest and the subsequent electroporation, are very challenging and delicate procedures. For instance, the optic vesicle of an embryo at HH stage 11 is difficult to distinguish from neighboring tissue, and practice is required to locate it with little effort. The glass capillary tube used to inject the DNA must be moved at increments of only a few microns at a time and held very still, which requires the use of a micromanipulator. The capillary tube must also be pulled such that the resulting tip is shaped to penetrate the tissue easily and release the DNA solution at a slow and steady rate. A small quantity of dye added to the DNA sample helps in visualizing the progress of the injection. As well, electroporation conditions are critical: there needs to be a balance between a charge sufficient to disrupt the plasma membrane allowing pores to form through which the DNA can enter the cells and embryo viability (Muramatsu *et al.*, 1997). In addition, the injection of an excessive amount of DNA into the chick's eye can be detrimental, as this can result in a non-specific small eye phenotype (Chen and Cepko, 2002). Finally, the embryo must be kept free of contamination from the outside environment, which requires cleaning instruments (scissors, electrodes) and the surface of the egg with ethanol, and adding antibiotics to the sterile saline solution that is used to bathe the embryo. Thus, considerable time was spent refining the procedure before we were able to achieve success with the retinal injections. Dr. Cairine Logan (University of Calgary, Calgary AB) was instrumental in helping us to establish this new technique in our lab, and Ms. Liz Monckton provided much valuable assistance with the injections.

As just mentioned, many trials were required in order to successfully infect a chick retina with a GFP/AP-2β RCAS construct. Fusing the GFP coding sequence to AP-2 proved to be very helpful. This tag eliminated the need for additional procedures (*i.e. in situ* hybridizations or immunohistochemistry) in order to analyze the expression of AP-2 post-injection, as the infected regions could be visualized directly under epifluorescent light. Twelve GFP positive eyes were obtained and preserved, and two of the best specimens were sectioned for further analysis. Preliminary results showed that in one of these two eyes, the retina was undeniably infected with GFP/AP-2β by the presence of the GFP signal. However, the infection was not as widespread as anticipated, occurring in a few localized patches in the retina. When comparing the cells in an infected area to those in an uninfected region on an H&E stained slide, mounted with a section adjacent to the one used for the detection of GFP signal, no major differences in cell morphology or distribution were immediately observed. It should be stressed that these are preliminary experiments, and they bear repeating before global conclusions about the effects of AP-2 misexpression on the cells in the chick retina can be drawn.

5.3. Future Directions

This work has opened up several avenues for future study, in addition to those experiments already briefly mentioned in the previous section. Of utmost importance would be following up on the two genes isolated from ChIP that showed promising results on the EMSA gels. Virtually nothing is known about clone 1213-13: BLAST analyses of its sequence revealed no significant alignments to known genes or ESTs in database archives. While this clone has been sequenced from only one end, the majority of the clone's sequence was obtained in that one pass. Attempting to sequence the clone from the other end is unlikely to provide enough additional information to identify the gene. Therefore, it is necessary to take further steps towards identifying this gene. For instance, one could use part of this sequence to create a labeled probe for screening a chicken genomic DNA library. Positive clones could be obtained and characterized, which would hopefully include much of the coding region, aiding in identification of the clone. Alternatively, a chromosome walking experiment may need to be undertaken in order to attain this information. Because some sequence data from the clone is already known, one might try designing sequencing primers to extend the sequence in both directions in a reaction with genomic DNA as the template. DNA sequence from clone 0206-17, on the other hand, was entered in BLAST searches, which revealed similarity to a chicken genomic clone (WAG-93J15) and similarity to various ESTs. Nevertheless, it would be desirable to obtain more DNA sequence from the region of the genome where this clone originated, so as to perform a more complete analysis.

The results of the EMSA gels make a convincing case that the two clones, 1213-13 and 0206-17, are *bona fide* AP-2 regulated genes, but it would be desirable to gather additional evidence that AP-2 binds to and influences transcription from these genes. A future experiment could include designing PCR primers to flank the predicted AP-2 binding sites within these sequences. Then, another ChIP reaction would be performed, this time with the inclusion of a PCR step at the end with the isolated chromatin used as a template. Bands of the appropriate size resulting from this reaction would be a strong indication that AP-2 binds to these genes. It would be beneficial to find a better antibody if additional rounds of ChIP are to be performed, as this may be fundamental to increasing the probability of success of future ChIP reactions. Also, if more ChIP reactions are to be performed, it can be speculated that retinal tissue at different stages of embryonic development (*i.e.* ED8, ED16) would yield different results. We chose ED10 as a starting point because AP-2 transcripts are abundant at this stage, but genes that are regulated by AP-2 at other stages may be missed. An alternative method would have been to perform ChIP on chick cells in culture, with formaldehyde added directly to the plate before harvesting, rather than on tissues, but it is doubtful if this method would have yielded better results.

More evidence to bolster the case that clones 1213-13 and 0206-17 are AP-2-regulated could be gathered after additional sequence data from these particular clones is obtained (*i.e.* through a genomic library screen). Once the complete regulatory regions of these genes are isolated, constructs could be made containing a reporter gene fused to these sequences for experiments such as chloramphenicol acetyltransferase (CAT) assays. These experiments would be instrumental in determining if there is a significant change in the activity of a reporter gene in the presence of AP-2, an indication that the gene of interest is affected by this transcription factor. As well, *in situ* hybridizations on chick retinal sections with labeled antisense mRNA from these two genes could provide additional evidence for AP-2-regulation by their spatial and temporal expression patterns with respect to the expression of AP-2. That is, would transcripts from these sequences be located, like AP-2, in amacrine and horizontal cells?

As for the three chicken genes identified as potential candidates for AP-2 regulation in the retina from the literature, the data gathered from the Northern blots were inconclusive. While this experiment did not show that these genes were affected by AP-2, it did not exclude this possibility, either. ChIP could be implemented to test whether AP-2 interacts with these genes *in vivo*. The drawback to this approach is that so far, only tyrosine hydroxylase contains characterized sequence from the 5' end of the chick gene. Choline acetyltransferase and dopamine β-hydroxylase have only some partial sequence from their coding regions available in the literature. Once the 5' regulatory regions of these genes are available, one could design primers to flank the AP-2 sites and perform ChIP. Alternatively, one could take DNA already isolated from the coding region by RT-PCR, and use it to probe a genomic library to find a clone with the 5' promoter region. This would be a labour intensive process, but regulatory sequence may be obtained that one could then use to design primers for a ChIP reaction.

Microarrays were not a feasible option when we began the search for AP-2 target genes, due to the fact that the chicken genome is largely uncharacterized, but this method may have yielded better results than SSH when comparing cDNAs from the two populations of cells that differed by the presence of transiently transfected AP-2. There is a growing impetus to sequence the chicken genome for economic and scientific reasons, since the chick is important for agricultural reasons and it is often used as a model organism in many embryological studies. Microarrays may now be a viable method to find target genes of AP-

2 in the chick retina. As well, it has recently been reported that investigators are combining microarrays with ChIP analyses (Weinmann *et al.*, 2002). Microarrays are established with genomic DNAs of a high CpG content, usually indicative of promoters, and they are probed with immunoprecipitated chromatin. Thus, target genes of a protein are identified in a high throughput manner. It would be interesting if the results of a microarray experiment designed to search for AP-2 target genes in the retina confirm those obtained through other methods.

A question that still has to be answered concerning the AP-2 target genes isolated in this study, once they are further characterized, is what their precise roles are with respect to retinal development. The *in situ* hybridization experiments already mentioned would provide some clues as to where and when these genes are expressed in development, which would be instrumental in forming hypotheses regarding their functions, in concert with their identities. It would also be interesting to extend this analysis by examining whether these genes play a role in retinoblastoma (RB), a pediatric eye tumour with an incidence of 1:20 000 births (Brantley and Harbour, 2000). To date, the cell of origin of retinoblastoma remains unknown, and this has been the subject of debate for many years (Kyritsis *et al.*, 1984). It has been proposed that RB arises from a cell committed to the amacrine or horizontal pathway, since the RB gene is expressed in the INL where these cells are located and RB tumours often arise in this region (Gallie *et al.*, 1999). Supporting evidence for this hypothesis comes from Robanus-Maandag and others who generated pRb/p107-deficient chimeric mice. The majority of these mice displayed RB tumours, which upon further examination showed eidence of amacrine cell differentiation through positive IHC staining with an anti-syntaxin antibody, a marker of amacrine cells (Robanus-Maandag *et al.*, 1998). We have found that a majority of RB cell lines analysed by Northern blots express AP-2 mRNA (unpublished observations) and we have shown that AP-2 is expressed in amacrine cells in the chick retina (Bisgrove and Godbout, 1999). As a starting point, we could use these AP-2 target genes as probes on Northern blots, containing mRNAs from normal retina and RB cell lines. A comparison of the patterns in the tumour cells versus the normal cells would reveal if the expression of these genes in RB cells has been altered.

As already mentioned, the results obtained from the retroviral infections are preliminary. To date, only one eye showed GFP signal in the retina following injection with the GFP/AP-2β construct and subsequent sectioning. While this retina displayed no apparent differences in cell morphology in the infected regions, the infection was not as widespread as desired. It is undoubtedly necessary to microinject and examine additional embryos before definitive conclusions regarding the effects of exogenous AP-2β expression in the chick retina are drawn. The reaction conditions may need to be slightly modified in future trials. For instance, it may be necessary to use more DNA in order to ensure a better spread of infection. A longer incubation time for injected embryos may also be required for the effects of the introduced AP-2β to be properly manifested. Alternatively, one could use viral stocks in the injection step rather than proviral DNA, which might increase embryo viability by eliminating the need for the electroporation. The effects of AP-2β might not be visible by simply examining the gross morphology of the cells in the retina. One may have to employ immunohistochemistry against markers of amacrine cells (such as syntaxin) to observe more subtle effects on differentiation. As well, only the GFP-AP-2β RCAS construct has been examined thus far. Now that the *in ovo* retroviral infection procedure has been established and that this construct is known to work, the corresponding GFP-AP-2β RCAN construct, as well as the AP-2β constructs without GFP, should be included as comparisons.

An alternative method for examining chick retinae following *in ovo* infections that could be explored is the use of flat mount *in situ* hybridizations. The sectioning of chick eyeballs, especially at a thickness of eight microns, requires a great deal of effort. Thus, analysis of multiple embryos can be a daunting task. We had opted to take several sample sections from various depths through the retina to facilitate this analysis, but in this manner, infected regions may have been inadvertently missed. In addition, it was sometimes difficult to align the GFP positive retina sections with the adjacent H&E-stained sections so that the infected region could be examined on both slides. Occasionally, the sections were not mounted in exactly the same orientation, or the retina became detached from the choroid layer during slide preparation, making a comparison between the slides difficult. In a flat mount *in situ* hybridization experiment the entire retina is contained within a fine piece of mesh while it undergoes manipulations. Consequently, the fragile tissue remains intact during the procedure, and it can be examined in its entirety. A couple of options would be available for detection of exogenous AP-2β in the chick retina. Because the construct we used contained sequence from GFP as a tag, subsequent *in situ* hybridization analysis could proceed with a probe to this transcript to distinguish it from any endogenous AP-2β. Alternatively, immunohistochemistry could be performed with the 3C2 antibody, which would delineate areas of viral infection as it recognizes a core protein of the Rous Sarcoma virus (Potts *et al.*, 1987).

One question that remains pertaining to both the search for AP-2 target genes and the *in ovo* manipulations, is the role of AP-2β versus AP-2α. To date we have concentrated on AP-2β. For instance, the approaches used prior to ChIP involved cells that were transfected with an AP-2β construct. Similarly, the chick embryo retinae were infected with a vector containing the AP-2β coding sequence. While the α and β forms of AP-2 are quite similar, their functions in the retina may not be identical. As the very different phenotypes of the AP-2α and AP-2β knockout mice have shown, these two proteins can have different non-overlapping roles during development. Thus, if AP-2α affects the transcription of a different repertoire of genes than AP-2β during eye development, these targets would be missed. Alternatively, AP-2α/β dimers may be required to regulate subsets of genes. AP-2β was chosen as a starting point for these experiments because its expression in the retina was more widespread than AP-2α (*i.e.* it was found in both amacrine and horizontal cells rather than in only amacrine cells). However, AP-2α should definitely be considered for analysis in future experiments.

5.4. Concluding Remarks

We have attempted in this work to discover novel target genes of the AP-2 transcription factor in the chick retina with the intention that it would lead to more insight into retinal development. We obtained two putative novel clones through chromatin immunoprecipitation, one of several methods employed, which will be further characterized to confirm that they are, in fact, *bona fide* AP-2-regulated clones. Preliminary results from *in ovo* electroporation experiments involving introduction of exogenous AP-2β protein into the chick retina have also been obtained. While definitive conclusions cannot yet be drawn from this initial data, the procedure has been established and several constructs are available for future experiments.

CHAPTER SIX

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Dr. P. J. Farnham: http://mcardle.oncology.wisc.edu/farnham/protocols/chips.html

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http://www.ncbi.nlm.nih.gov/BLAST

OMIM™—Online Mendelian Inheritance in Man (Johns Hopkins University):

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM

TRANSFAC database: www.gene-regulation.com

Upstate Biotech ChIP protocol:

http://www.upstate.com/misc/protocols.q.prot.e.chips/Chromatin+Immunoprecipitation++ChIPs++Assay+Kit

APPENDIX A

Details of the ChIP clones analyzed by EMSA gels

Clone 1205-3

BLAST search results: No significant alignments versus nr and EST databases

Predicted AP-2 sites: One AP-2/Sp1 site predicted by Tfsitescan

Sequence:

tgccaaactctgttcactgagtttcataatggaactatgaaaagggaggtgtttcaaatgtacatatactccctttagtttcttgcaagccctg
gtatatccttagctaaaacaccgtcttatgtacagcagctgtacttgatctgtctctgttttgcataactgcacnaaattaaaacatgacgaga
gtagatgccagtgttttttttttaccttgtgtcccagcttatgaatgatcccattaatgttaatagycartttcggcctcactttcaaaactgact
tttgtatgtatccg

Clone 1205-4

BLAST search results: No significant alignments versus nr and EST databases

Predicted AP-2 sites: Several sites predicted by Patch

Sequence:

cgggctgtgccacatgccgagaaatgcatgccagcaaatatcccttctcatttcaattaccgtagaaggagtagcttcacaccgttttctctg
aaatcatgtgtgcagtgtcaggtaaggcaggatgtgactcaagcatgactggattcagaagcaggaactctgtgaaatcttctggtttcagt
tcccagagattttggttttgtactttcctccagacctgcacagctctgttcatggggcattcttgggactgctgcctgnagtgancaaaagat
gnttccatannagctgctctaaactatgnttcggtcttctatcatacctagtcctctgctagagaaaagcnaataagagaaattgattaccct
ctgcttcctattccccctccttccccnatctctgctggatac

Clone 1205-22

BLAST search results: No significant alignments versus nr and EST databases

Predicted AP-2 sites: Several sites predicted by Patch

Sequence:

taactttcaataatttgtggttcacagtcactatgagaaatatatacagccacttttcagaaaatcagattttttttaataattcagaaaagtgaa
cattgtatgtaaaagcagatttcatattgacaataaattactttttctttttgcctgaggtactataaagcaaaaataatcttataccaagnttact
tttcaaaactcaaacagttaanatgttgattttgcagaagctttgctgaatgatgaaatgatctctgggatttaaaactgtctgctttcttgacat
ttaaaanacacttttaaattggtaagaatancaaaacaatcagcaaacgctggcanataatatgncttattctgaagcnaaaaccagactc
aanggatnggtttgtctcccaaagaaaana

Clone 1205-24

BLAST search results: No significant alignments versus nr or EST databases

Predicted AP-2 sites: Patch predicts several

Sequence:

cggctcctttcctttttttttcttttttttttttttcccccagtcccttcatttatgaaagtgattatattgtgagcaggaaaaaaagggaactttg
aaagtttgtaggaaactggaataaactgcatttgaaaatctgggaaagtcaggagaacgtgcaacatattttcccttgataaaattgagaaaa
nnanaaaagtactcttgagacatatgtgtttccttctgctgagtgacagccttaaggacagtacatgtgagagaagttttagaggtaaaaat
atttttcttttaactgagtagcagagaatcaggggtgtttgaagcacatgtaaatnggcagaacagnctttgnctttctgggtttttttncct
ctc

Clone 1210-1

BLAST search results: No significant alignments versus nr and EST databases

Predicted AP-2 sites: Several by Patch; one by Tfsitescan

Sequence:

gccctncgctctanaactagtggatcccccgncgctgacggtgagatgaatgccaaacgtggtgaatgttagcccacaaaaaccaagtga
gtgggaagacttaacatgatggctggatgggaataagaaagaacaaaccatgaactcaaaattctcagctcctgtgtggaagcaagaact
ggcttatcatatttggaaacttctgcaattccattaagggctcttcataataaccaaatatgcattgctaaagcaaccatggttagtttaaaata
tacttgatggcagtgaaagctagaagtactttccctgctttccctatagcattcattcagatactgaagaaaaaaggtgattatagccaaaaa
naaaaaaaaaggnaaaagagtnattcttacttcagcactgatttgaatacctctgatttanacccaac

Clone 1210-5

BLAST search results: No significant alignments

Predicted AP-2 sites: Patch predicts one

Sequence:

cggactacatttgtcaatcattctttagtaatgataactcatgaacagcttctataagaagagtatcaatattagagattaaacagggttgttac
ctcagagtagctcaaatataagtacagtattgaccaagtgctcatcatcatgctagcaaaacaagcttttaatgaaagttatcatctgaacat
gattgggaagttaaatgattcaacaaaggttgctttctttccttcttttctattttctctttcttgattactttcttggccccccctgtaagaaatttag
acaactttttg

Clone 1210-9

BLAST search results: No significant alignments versus nr and EST databases

Predicted AP-2 sites: Tfsitescan predicts several AP-2 binding sites

Sequence:

tggaacaaaccttgggggaaaagwgttcatttaaatgtttttcccttttcgycaacttcaacgattctttcaatgtttggggaaggatcmcca
tttataaacaatatttttngntnncctgccatgtgagcaatacagactaacagtcagcactgagcanyaacaaagaaatgattggcttcatt
acagctcagtgccagccaaggagttgctgytactttctgatgctgttammagttccagttcatatccagtggagatatcmmtgmaam
aagcgcttytctaattatcgcgtccyctgwcctgtgaacatgaaaaataatttyccctygycmtagatcaaaawymcaantgcatawt
mtmtwmcamcttttatgtcmaayagaaarkcmtttngcctttncgagnawactatttwwwaaaatmycccttcctttccccaty
tyaawaaamattgcc

Clone 1210-15

BLAST search results: No significant alignments versus nr and EST databases

Predicted AP-2 sites: Patch predicts several; Tfsitescan predicts several

Sequence:

tttacaggcgaagtttgatmtgaactccccagyggaaacgccctgaatagtaaacacaaamaaagctgtctttaaaaagatwatacttat
tttactccaccaatcttacaggttctgttgcaatgggccttagaaaggcagatgtaatcctagagtcatccccatctctcagaaagctcttcc
aaaaawwctaaataaccagaccctgcaagacgaagactcmcatggcactgggttgaatgggyggyaggtgggagctcttgcagctcg
aaaaaaatymagtgatttggygaatttttactcatgaatgcyatnmtataccatcatgtctggtcacacttacmagatggctcmwtact
ntgcaarntgctsagargcwgtggtacccaatcctgtankttt

Clone 1210-17

BLAST search results: no significant alignments to nr and EST databases

Predicted AP-2 sites: Patch predicts one

Sequence:

ctgsaaagaaaaantccaggtcagtggcaagctgcttaaagagccctttytyccnggtgamtccaragragmcattcctgcaggtttaa
tgttacagtcccgatctgccactgcttttccctttggtgctctcaccccatgagacaagttgctggagaacaaccaggatttgcataaaccgt
gtgtgtgcacaactttgatgtgcattgttgccgctgccttggtgtctgcctttgaatatttgaaaaggtgktaayaagtcaatcaaaagccagt
taattcaaatgaaagccagcttstggtgctyaayacaccctgttatatnttgtttgtcattatctctcaggaccagtgactcagccctgtaaaa
rgnttcaaaatgatcacccg

Clone 1210-18

BLAST search results: No significant alignments versus nr or EST databases

Predicted AP-2 sites: Tfsitescan predicts several sites

Sequence:

angtttatctggctacatgyactattctcctycctggtatttagtactagyyttgtttatggsgagagaattytaaagagctaatcyttcaggat
gacaamatcagtacactgnnnnatagcagttatttaaaaattgatctgataatgattattgtygttagyctttgattggtgatattaattasa
atagtttgaagaaagatgatcmataatgtggaattatataatatgctatattgctttaygaatytcyttaattyyctttanagstataaattgcat
tatgaatatygcgtcymarsaatamcataarsccmatttttywggyaggygcttyctcmcmcaaggmattcctcttcttamcttgaa
aaawwmwggatsksccrsccnyyytgggtymmykgaagaacmctcnscnwnaatymacytgaangtytanaaactcacmn
argctnmcnc

Clone 1210-22

BLAST search results: no significant alignments against nr or EST databases

Predicted AP-2 sites: Patch predicts one

Sequence:

ymtttacagtaaaagtaaaaaaagtaaccttgtctttcctagagaaggttgcactgatttcttttcccttaatttcataggacatttaaagag
gaaaggaaatgctcaaaagtaaaaaaagattggctgctattcagtgtttttcagtctgcttgaagtgctgtgctctgcagcacrgcttcccct
gcaaaagaatttcctgtgagcattgggamtgaattactgcgacamttctgggttatatcccaataatagttgaagtgatcctgacatacaat
aatasyttatataaaca

Clone 1213-6

BLAST search results: No significant alignments

Predicted AP-2 sites: GeneTool predicts one; Patch predicts one; Tfsitescan predicts two

Sequence (from both ends)

ccgnnacgcgccaannnaaccgaacnnggaaanagggggatcaacgggggtnnccccctgnanagaanagaaaaagganccttt
nccctgnaggaaaannaaantaaaggctgacctgttcatccctgcacagaaggatggccttggaaggcttaggagattctccncttccc
aacngcnngnnaagacctccaaatacttctacaggatatatggaaaaccagtgctagnnaacacaaataattacagncanaaatattgg
gcctntcanccanaatnggngcaataaaaaaannggcccgggctcggnacaaactggncaatatgncttcccnttcaaaaaannaa
ccannntggcagttcttncttactaataaaagggnaaggnccaaaggccacnaattantgaaggntggacaaaaanggacgaactgg
aaataacggccaaaatcntgaanntcaatcccnaaaaaccccntgnaaggntngggaagggganaantatttgcnaaaaantcntccc
cntaccctttgggctaagnaatnngaaatcaagncttancggaaaccggccgcccntccgaggggggggcccggaa

Clone 1213-6 other end:

aatcgataagsattgatatcgattyctagamaaagggaaaggaaagtattgcnamtatttcctgacagcttcagggattttagatgaatcasattagcatattcagttgtctttttgtcaactcatattattccttggnatcacttaataaagaagagtgcmaaagataattttgaaggaagcaatgacaattgtacaaacaggacaatttttattcccaatctggtgaaagccaatattatgctgaaattattgtgttactagcctggtttccatatatcctgtasarrtattggmggtcttaacagcagttgggaagaggagaatctcctaagccttccaaggccatcctctgtgcrrggswysammaggtcagcctttagtntygaattcctgcrrcccgggwggatccactagytcwagagtggccgccaccgcggtggarctccagyttttgtycccctttartgrrgmsyttaattycgascttggcgc

Clone 1213-3

BLAST search results: No significant alignments

Predicted AP-2 sites: A couple sites predicted from Tfsitescan

Sequence:

atmtkcmttctgaagygttcagacactgacttaacacmgacttagtctatccasgcttttactggagacngattcttgcttttacctactgcagctcctactgctgtccagccctcnttatccwgnncagctccttgtctatcgcagactaacmmatgcctcamttctatgttaaaaaataataatcatcatgyytttaaaaccagaaataacagtcctttcttccttccctgcttatagagagcaacgtgamcactcaccacctctcctcccctcycccamctacaagattttaggamctactaccagaaggtcaaaargargyctctgcttttgtaacaanygctattttawgaaaaaattgctgmctgctgcatyaatgccaawanctgcatatamytttarsntcagcttssyaaaattntaaggntcagatmcmaagg

Clone 1213-4

BLAST search results: no significant alignments to nr and EST databases

Predicted AP-2 sites: 3 predicted by Patch

Sequence:

tttcatattgctcttgtttatttacatcaaaaaattcaagtatgcattttaaaatcttaaaacatacaaagacttgtaattatttcctcatagataggcaaaaaggtttttttaaataattttatttttaccctgggagtcaatcagagccggatcttccaagttaacagaatttaaaatatatgagccttatgtgagccagaatctccaatagatcagtttgtcatatggtcacttga

Clone 1213-9

BLAST search results: No significant alignments

Predicted AP-2 sites: AliBaba2.1 predicts one; Patch predicts 8; Tfsitescan predicts two

Sequence:

tmmgcagagcgagtgcaaacactgtaaatgtgcagtgcgctctcagctggggaggataagatggcagagcaattgttgtttcaacagtatagggaagagaggtttggaggtggatatattttctttgccttactctgtttgaatgcacagaaactgggagtcacggtaatgctcttgtaatctccatgncacgyctgggnanakscatgggggtaananccccttttttttyytwknaatttggatttcnnmataantttrcagawaaaactgcawaaatttagcagawttgcctatacaacmnctcganngctctatttccatcctcgggaagctatttttttgtcccttggctcmggga

acttattttgatcasctttmaggtaatttmctttgttgggtcaaaatggaangaaratgarmcaatatctctytcttttmrntgcwkcctaa
agggaa

Clone 1213-13

BLAST search results: No significant alignments

Predicted AP-2 sites: Tfsitescan predicts two; Patch predicts several

Sequence:

gaattcaattcagcaaagaacaaaatagacaasctaaaggcagtcagaggcnkknmayyatttgctttattaaagttgaagcatttgctc
caaggagataggctaatgaacctccaatctgnaaggctggagaagatgttgagggaccaatgcmcagtctaccaatgcctgtaaggcaa
caataagacggagccargccctccactcaatctcatgatgtgagaacaagaaacagtgaccataaattgywwkaggagattytcagam
tgcatatmmmtctggagggtgtaaacccaggaacaggytgyccagagatgctgwagaaactgctctccttggarggcttttnaaactg
maaatgmnaaaatgnccctgnaggcaamcttgstccaaaattcagggctgamcaagarscccccctcctaawgtngatgggttntgt
cttggratmmtttytaacccaamcat

Clone 1213-2

BLAST search results: No significant alignments versus nr and EST databases

Predicted AP-2 sites: Tfsitescan predicts one site

Sequence:

acttctcagctrccantcamgttctttcttgytcatgtaatcgtcggggattctatttcagggttaccttttgccttgtccagyctgaaaggtct
gacactctgtttgggatacannntcaaacaaggactagtccaaaggactggaaacttcacattagnatcatggagttcaagttcatttgcat
cccaygtgagtcaatttaacatccatctcagccttgagctccaagattatccaasaarcttccttctgccaagtcaktgagctattggaatttg
aatttgatgttccttgctatcatgyattctccttccggaataaaggcascttycwtkgctgycawcytcyytgnttastaaawgaaawaaa
tttgtmccctctgntaytcctycyttgmcttatctcaaarcaraymttyyttasawg

Clone 1213-19

BLAST search results: No significant alignments versus nr and EST databases

Predicted AP-2 sites: Two predicted by Tfsitescan

Sequence:

tatcyscnyncctgttmaaaggaggaagcmatgtymtattanggagaaaaatgaggtgaactactyccaagataagatttctagtaac
atttttaamcammttacttctcamacttgsgatgcaaaatyttnnctcmacattttagtttgtaatatmcnagaamatccttgtgyaaa
atgcatnnagtatattckacagmyaytagatctccammmtgttatgcngctynmsyatctytcanttgyamnsatgtttagantyta
skacaargaatyattgtyctttctacataaysmmctaacagcyatgcyaawtgcyyyttakwgcmamamtgygyggyawggyaat
yatanamntgtcmtttnmmatggaassctatmatyaymtgsyycmtmatttmgawascttgtymammctgwattngmtyg
aaaycccaaatycatgsaawcwcntacangcwtggcamsgggmcc

Clone 0115-2

BLAST search results: No significant alignments

Predicted AP-2 sites: AliBaba2.1 predicts one; Patch predicts one; Tfsitescan predicts one

Sequence:

gagctcagcaaaaacaattaaggcagtcattttaagtattaatttctgatgntggnggctgcctgtgttcggaggtcacacttagttgaagca
cactgcgcctgtggaaattctgctgagcaattagcaaaaataccccttgtcattaggaagaaacaagaacactgttctccataaagnaatcag
tggcgtttcagcaagtgacacctctttgttttacaggagaaaaanactacagnatgagcctaacatattantttatctgagaaacaaaacgtt
agganggacaagactgtgcttgagttttcccagtaatgagtgcct

Clone 0120-4

BLAST search results: no significant alignments to nr or EST databases

Predicted AP-2 sites: Patch predicts several; Tfsitescan predicts one

Sequence:

cggggaaccttccctgccctccttgttaagtatttagttgcagagttgtccagactgcttaattttctctgcagatttcaagggccttttcatgg
ccctcatgatgacaggaagcatcatacaacaggcacagggagcactgaagatttttgcaagtgatatagcatgagctcaatactcatgatat
gttttcctaaaaatccctgttgctttcccagacctctgcctgctgggttggctttttctggggacacctctgcatgnggttaggcctgcaaca
gg

Clone 0115-4

BLAST search results: no significant alignments to nr or EST databases

Predicted AP-2 sites: One predicted by AliBaba 2.1; Tfsitescan predicts several; Patch predicts several

Sequence (from both ends):

aacnnnnacagnggcnnanngggaaantggagngtcacgggtgnnccccgcaaaananaaaaaggancccccnccctgaagg
aaggaanagaggtatagaaaggacaactgaaggattaanagactccgcaggagtcnatctcttgagaagatgatgaagccaggcctctt
tgattcaaacgtgaattggagaggnggaanatattcagagctcacaaaaacctgatgacagcatataacatccacattgacttgcagaattt
tcntgcanagctgttacctcaagggncangagcagatactgaaaggaacagctgatgatgcagatnctaancaatgacaaaagctaga
agaggaaggaggantgngaagcaatatcnacgngcnggnnactancactgcttaatnaactgctgatctcngaaganggaaaaacn
cggcnacangnactnacagnaccggacanagcagggtgaaccgnnacaactntcnaaatagaaaactacttggaggaaaaaannn
gnacgg

ansrawggggwccggggccccccycgannyyyymggwawmgawaagcttgawtatcgwmtycccgtgagatgacatcaac
gctggtcacacaagctggggagtcagcccacttctaaccggggctgcctttgctgacatggatgctttcggagatggagcctgtggcaca
gagttgtgtgtagctgtctgggaagcaagtttttcctgactgtaagtgcctggagtaagatataattgagacagctaaacttgacttcttcctg
ctctttctcccataacccaaaacccaagttcctcaaccacatctagcttagcatctgyccttttcttctctgaattgatgtccttgaaggagtat

aacggaaattagttcattacatcscatcgaraggatttcctgttgttaaaycaagcaatatttcgcagtcaatgyttctcctcaagayrtttcgatttwgaaggtgaaaggtaaccatamtgtgtcargycagtaatgsmwktagwcaatatnccatctn

Clone 0115-7

BLAST search results: Significant alignment to Bradyrhizobium japonicum DNA, complete genome (nr database)

Predicted AP-2 sites: AliBaba2.1 predicts two; Patch predicts several; Tfsitescan predicts three

Sequence:

gcggccgcgcatcacgccgaaacgcgggcgctgctcgtcgcggaacttccactggatgtggyagaggttgagcaggcaggytcttgyaggacttgacgtaggagcggaagatctccgtgatcatctcctcgttggtcggtccgnacaggagctcgcgcttgtggcgatcgaggatgcgcagcatctccgggccataggcatcgyaacggccgctctcacgccacagatcgscaagctgcancgtcggcatcaggagctcgagcgcgccggygcggwcctgctccctcscrgaacgatctgctcgatcttcttcagcacccggaagccgagcgggcarccaggcwatakatgccggnggccytcctgccgcwtcatgcccggcgmgcrgcwtcnrccggtgcgaaackayctycgccttcttc

Clone 0115-17

BLAST search results: No significant alignments

Predicted AP-2 sites: AliBaba2.1 predicts one; Patch predicts one

Sequence:

gaattccggggctgtactacagccctgacagcatgctccctgttcttaaaaaataaacaaaatctctttagaaaatctatctgtgaacattggcttctgcaggaaaaataatgattacataccacttgatattctggaatcagaaatatctcttctcgatgagcgttaaaataggaaaatgactgatagtgctaggttggattgttgttgtttttaatgatcttattaagcattattccatgtcccatttttttgtcagatactactgtccatttacagaaatgcaataatttcacctcattggcattctttttttatgaaagctgaggaatgcagcagntggctatggtgatttaggtggcagggtacccgctggctttctgtaacctttggaaaatncttcaattctttctcttcccctttgtctactctgaa

Clone 0115-18

BLAST search results: Significant homology to human dihydropyridine receptor alpha 1 subunit, human DNA sequence from clone RP5-894H24, and various calcium channel voltage dependent subunits (nr database)

Predicted AP-2 sites: 3 sites predicted by Tfsitescan; one by AliBaba2.1, 7 by Patch

Sequence:

gaattcaataatgtacaaatgggataatggcttatgggggcccatccttccctggcatgctggtctgcatctacttcatcatcctctttgtctgtgggaactgtatcctctgatatcaaggctgtgtccaggctccctctgcccacccccatagactgctccagctgcacaggactgtgcagattgctctgtgtgtgtgtgtgtgygtgtgtatgtgtaaggactgagctgaaggatggggccatcagcccagagcacttgaatacacagggtnagggatcactacccaasggctgtammaagggctcatctgcaggarggtttctcagagcaaagcaganactgtgggcrrccctggccatawcctccaammattgccaagattcatgtccttattgctgaccctccagaatwtcctcctcaackyctncrtgg

Clone 0120-5

BLAST search results: No significant alignments versus nr and EST databases

Predicted AP-2 sites: Patch predicts several sites

Sequence:

ttatttccaaaacacagtcacaaggcagagcatataagctttagaagcacttctgatcaaacctcaagtgactgccaatatttggaaagaaa
gcagcaatgttttgtttctggtaaaccattgcacgctagagcttgcttctcaaaacactgaaataaatgggcatgcatgcaacaattaatcttc
ttggttatttgctacgcaaccagatatgcaaatcattgatcctattaaagtctatggaaaatacagtagtgtggtaaggaaaaaaaaaagagg
cattagtactaactggccagtaaagctttggggtatgctggtttgatcaataagaatgccgttagattgarttatgatgatatgtggcagacaa
atttacacagtttgargcatgacaacaccaaggatttcagntaagatggggtagatgaaaa

Clone 0120-19

BLAST search results: No significant alignments versus nr and EST databases

Predicted AP-2 sites: one predicted by Tfsitescan; several predicted by Patch

Sequence:

cattactaccgttgtgaatgtttctgtacagtgaaattccaaaagaaagctttggttttgtttgggaggtttttgtgtttctgtgtccaaatcagc
agttgtttttttccttctaagcacattgatagctcatttttggcatgaccaccatcttcatttgggacttgatttttgcctttgtgaaatgtgtca
tagccagtttgtaactgaaagtct

Clone 0120-11

BLAST search results: No significant alignments

Predicted AP-2 sites: GeneTool predicts one; Patch predicts several; Tfsitescan predicts one

Sequence:

ycntaaatcctggkaaacaataaaaattaaatgagttcttttggaattgtcacwatcstggtaactgctttgctgctacaaatctaaaacaaa
gaattaaagttaccaaaataaatgagatgttagggactgttctttttccccccccaactccctctccttaataacctgagatgaatcacagt
ctgcttagaatttcttaatgcttaaaataggttgcagaagtagggcagagacatgagcatacatttagkatatttgcattggccttcaaagta
mnctgmtttggcaaaaaagaaaagggmmsaaagtcaccattgtcctggaaattcaattctcct

Clone 0120-22

BLAST search results: No significant alignments

Predicted AP-2 sites: AliBaba2.1 predicts one; Patch predicts three; Tfsitescan predicts two

Sequence:

cgggactaattagtaacactgtcccatcagttccatttttaagaaattgatttttttgtttgtaattttgccaaacttcagccattttgattgmm
mtttcttggtctgtaggctgatcaaaagcnngtgnataaaaaccaaccaaccaaccaaaactcttatggattagaataaaggaaagtactt
tgttccaacattttaaaagaaaaccagnggattacctttctttctgaaaattctaggtttttttccctgcttyngraggagggcttgaaatgta
stmmcssytaynakaaaggaaatgtgacactcnsagaaamcargtgtgagtagaatggggaaaaaaagatgagaamctgaagaag
aaaagaactagyaacctgggsctkgaantagctaatcaaaatgargaarrmctcaaaatttggaaaaaggrcagagagacwacmttyc
agaangagganggrcctatgattcctgmgccg

Clone 0115-9

BLAST search results: no significant alignments versus nr and EST databases

Predicted AP-2 sites: one by Tfsitescan

Sequence:

tataaaatcmagtaamtatttccattttccaactcaataacatcaaactagtgaaaaygattctgtaatttttatacaaatacatgcmamca
tgactamatygttaaagncactnntgaycataccaactgattttctcagaamacgytctgaaaaatggaagacatatyattaatagaatta
tttacgtaggaamgtacaaaaaaaaaatcwmagagcatttcagacnagctcagactttcttgyarcactatatatctgggagtytctcyy
ywkamntctgcmctgcatmtctttcacaacmtcagtttgtgtctttatasaaagaatyntgymcttctaatgaaaaattttaacmtcty
wanttttyaaatctttawcctcctgcggcaaactycccnyttcctmatcnygcctctmcawtt

Clone 0120-2

BLAST search results: no significant alignments versus nr and EST databases

Predicted AP-2 sites: one by Patch

Sequence:

cggagctgagatgaagactggagcaactctcagtgcaatgtgagtttcacatttggggcccaacagggtaagaaattaggaagattccaa
catatatgaagagtatgttcagactgcagaaaggaatttttcaaataggtttcaaatatgggcagtagaaatgagagttctgagacaacaata
aaagtagctcaggtaacatttatggaaggaattggtga

Clone 0206-17

BLAST search results: Shows significant alignments to *G. gallus* clone WAG-93J15 (nr database) and several *G. gallus* cDNA clones (EST database)

Predicted AP-2 sites: Tfsitescan predicts one; Patch predicts two

Sequence:

gaattctgtgctaaagaagacctgatctgaaaaacattcagacaattgtctcataagcacatgtgcaatagttctcatagtgtaaagcatgca
tgcccaaccttttggcttacctgggccacaatgagtgaagaggaattgcctagggccacatctacaaatcttgctctgaaaataatgcctaa
tagttatttccgtggaaacaacagatacagagagcacagtatcactagttagtagagcaaattctcaggcacaaaacactccggaattc

Clone 0206-23

BLAST search results: No significant alignments to nr and EST databases

Predicted AP-2 sites: Patch predicts two sites

Sequence:

ccggaatataagcctaagaatataaaaatattaactacttcattctctcttttgwkaacctctaagaagtggccgaatggtctaaatctgcag
yytaaatgttacttgctgtactatctatgaagtatttatatccaaatgcctgtaatgcagatttttcttgtactgtttgcattgccgtgtgttttaaa
acgsmrgtggtagtgmnattggggtttgcagctatgctgctctgtgaaa

APPENDIX B

Details of selected clones obtained by ChIP excluded from EMSA analyses[*]

Clone 0206-8

BLAST Search results: Aligns to *G. gallus* cDNA clone chEST853n9 (gi 25826890) in search of EST database

tacctaggccctctgcagggagaagacctgcgggaagacactggagagttcagttgaactagatgatcttagaggtcttttccaaccttan
tgmytcaatgattctatgtcatggcagttgtcctgtcccgtggctgcaccctggagttgtcccgtcagtaactggtcagttwatactgtctc
gaaggtttaaaccagcccmctcctgggccatttgtaaagagtggnaattaccatagctaagcagagggccgtgyatctgaatagaagtc
mctctggaatamgsyaawngtctagtttttcrgctaatggggnnaagaaggcaagtcctgagatttaagyaggarcwrggmctcaga
ntaccacagattncttttyctttttctcctcttcmggtactaaatmmtgnacamctcagagggggtgaatacatnagwkgcaaacgc
tgccgttmttgyctccycmacaaattgaggtggamtycaaaaca

Clone 0115-10

BLAST Search results: Entire sequence aligns nearly perfectly to *G. gallus* cDNA clone CSEQRBN09 (gi 25918971) in search of EST database

cggcatcatcatctgcaagcactcagtcctacagccaacagcagttttattgctgacttcaaacgggagcagaattggacccaaccattca
aatcccctcacacagtgattatctggaactaggatccaatcatttcatggcagatgaagatgtctgcagattaatacctgcaaccgttcacag
aggagtgcaaactgcttcaggtttactgctcaatacagtaagctgtcatttttagcttatacattttctgctgyaatttaaggacctccaaaag
tcagcatttaatcagggctttcctatgagctcccrrgcagagccacagagcgacccctgaaatgctgtgccamamagcgacscatttac
aarcgctgmgtggaatcccarctccactgaamcmcamagc

Clone 1205-16

BLAST Search results: Entire sequence aligns nearly perfectly to *G. gallus* cDNA clone dkfz426 (gi 7120576) in search of EST database

cgggttcattattcttccaagtgtaaacatttctatattaaataattttctatcgtgtccacgtagtgcatggaatgtttgtcagaagacagttct
ctaagactgtttattaccaagttagcaaacgtaacgcttattaatatcagatctcagtagtttaaaaatgatttgccttaccttgcacaagtaaa
gcagggtttctgaaaactgccttcagttatctttccttaagtataaa

Clone 1205-14

BLAST Search results: Aligns to various clones in search of EST database including *G. gallus* clone WAG-68G2 (gi 19747195)

ttcttagaaagagtggtgctgcactggtgcaggctgcccaggaaggtgttgaagtcattgtccccagaggtgttcangaaacgtgtggatg
tggcactgatggacatggttaatagacatgtgctgaccattggactagatgagcttagaggtctttcctaatctaaatgattctacagttccag
agacagtgctttgcacatgcttgcacaactttcccacaagttangannacaaaactccagagctgcatgaaagctatccaattaatccaaa
cattgaggacangatcaaataaagcttcatatgcattctctctatgggtaaatattttaggatacaatcacagctttacaaaggcaacatgag
aaacagtattttccctaccctctgaangctcagtgcactaatacc

Clone 1210-4

BLAST Search results: Aligns to human DNA sequence from clone RP11-184D20 on chromosome 1 (gi 15795523) versus nr database

tcyccattattgctgttaaataatgcataaatattctcccatgctgaaatacttctatttcctctccatgaattattttgaatgtatcattcagtygy
tgttctatttaaaaatgtagttagaactctgcatataacagtattatgtctggaagaagtatcatcacaagtagttggctaacactccatgctgg
aaatgaagtatacattattaaawggggccatctccttccttcttgggaaagsattatcaccagaaatacnatatttagnccsg

Clone 0115-20

BLAST Search results: Aligns to R. *norvegicus* and H. *sapiens* mRNA for utrophin in search of nr database

agccaagaggnaagcatttcagcccaccgagttgacaaacgggagcccccacattcttaccaaatgactcaattcctcatagcgggcatt
gaaagcctccaatttttcactgatgatatcatcaagaatccctccatcaatcaacgtctgcccaagttcccgaatctgggttcgattatcagct
ggatgacgcnggactgattccagagactaatcagaggaaaaggttgcaaagtcaatgcatattgtaaggncattgaatatttctgaactttc
agangtagaaatctggncgcatgcaaagtttacagtactctcangacaaacaagnataaagaagtatgaataaattatggcnttaatccat
gatggcacanngtaaaatacaagcnanttgtttttgtttttncataacccaaactttcaacangnacttcttntancnn

* These clones were excluded from promoter and EMSA analyses, as they appeared to not consist of sequence from gene promoters following BLAST searches. They either aligned to an EST clone throughout the length of their sequence or aligned to a sequence that was in the middle, not the beginning, of a gene.